THE APPEARANCE OF AMINO ACID TRANSMITTERS, THEIR RECEPTORS AND GAP JUNCTIONS IN THE DEVELOPING CHICK RETINA

VIOLA BONNESS

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Department of Physiology
University College London

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Abstract

In the developing CNS the majority of neurons migrate to their final position, further differentiate and form synapses one with another. This highly orchestrated process requires intercellular communication from earliest times. This thesis investigates the appearance of amino acid transmitter release, the expression of amino acid transmitter receptors and gap junctional connections by ganglion cells in the embryonic chick retina.

The release of the amino acid transmitters glutamate, aspartate, GABA and glycine from the retina was investigated using high performance liquid chromatography (HPLC). These experiments show the release of all four transmitters can be detected by HPLC from embryonic day four (E4) onwards, although in the case of GABA such release was only detectable when extracellular potassium was raised. The ratio of release under normal ionic conditions to that evoked by raising extracellular potassium increased towards synaptogenesis. High potassium evoked both Ca$^{2+}$-dependent and Ca$^{2+}$-independent release of transmitter.

The developmental profile of the appearance of receptors for the four amino acid transmitters in ganglion cells was established in situ by whole-cell patch-clamping. The results show that ganglion cells respond to GABA and glycine at E6, while responses to AMPA and NMDA appear at E6 and E8, respectively.

The time course of the development of gap junctional coupling between ganglion cells and other cells has been established using whole-cell patch-clamping as a means to introduce a mixture of gap junction permeant and impermeant dyes into their cytoplasm. Confocal microscopy of these preparations shows a general increase in the extent of coupling towards the time of synaptogenesis (~E12), which is followed by a marked decrease at E14. Throughout the period examined (E5-E14) some ganglion cells were
coupled to cells that traverse the retina, which may represent neurons in the process of migration to their final destination.
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O Fortuna,
velut luna
statu variabilis,
semper crescis
aut decrescis;
vita detestabilis.

Nunc obdurat
et tunc curat
ludo mentis aciem;
egestatem,
potestatem
dissolvit ut glaciem.

(From: CARMINA BURANA)
Chapter 1

Introduction

1.1 Organisation of the retina

The retina has many advantages as a tissue in which to study neuronal development compared to the brain. It is easily accessible and its structure is well known. The signalling pathways in the adult retina are well established and much research on retinal development has been carried out already, providing an abundance of background information.

1.1.1 Basic Anatomy of the retina

The vertebrate retina consists of three layers of cell bodies: the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL). The dendritic processes and axonal terminals of these cells are located in two layers of neuropil; the outer plexiform layer (OPL) between ONL and INL, and the inner plexiform layer (IPL) between INL and GCL (see figure 1.1).

Five different types of neurons form the retinal circuitry, organised as vertical and lateral pathways. The vertical pathway is comprised of photoreceptors, bipolar and ganglion cells. The photoreceptors lie adjacent to the pigment epithelium and their somata are located in the ONL. Their terminals form synapses with bipolar cells in the OPL. The bipolar cells, whose cell bodies lie in the INL send axons into the IPL where they synapse with the dendrites of the ganglion cells. The axons of the ganglion cells run across the retina in the axon fiber layer (AFL) and form the optic nerve which carries visual...
Figure 1.1

A schematic diagram of the anatomy of the adult vertebrate retina.

The photoreceptor cells (PC, red) are located nearest the pigment epithelium and their somata lie in the outer nuclear layer (ONL). The usual signal passes from photoreceptors to bipolar cells (BC, yellow) and from them to ganglion cells (GC, green), the axons of which project into the brain. Lateral interactions in the outer plexiform layer (OPL) occur via horizontal cells (HC, blue) and in the inner plexiform layer via amacrine cells (AC, orange). The predominant glial cell type of the avian retina, the Müller cells (MC, lime-green) span the whole thickness of the retina with their somata located in the inner nuclear layer (INL). Some amacrine cell somata are located in the ganglion cell layer (GCL) and are called displaced ganglion cells. Similarly, some ganglion cells are ‘displaced’ and found in the INL.
Anatomy of the adult retina

PIGMENT EPITHELIUM

VITREOUS BODY
information to the brain. The lateral pathways are comprised of horizontal and amacrine cells. Horizontal cells mediate lateral inhibition in the OPL and have their cell bodies in the distal INL, while the amacrine cells, some of which are responsible for motion dependent lateral inhibition in the IPL, have their cell bodies in either the proximal INL or the GCL. Those with their cell bodies located in the GCL are called ‘displaced’ amacrine cells. Similarly, there are ‘displaced’ ganglion cells found in the INL and ‘displaced’ horizontal and bipolar cells found in the ONL. Unlike the mammalian retina, which has both astrocytes and radial glial cells called Müller cells, the chick retina contains only Müller cells. Müller cells extend processes to the outer and inner limiting membrane and have their perikarya in the INL.

1.1.2 Information processing by the retina

Most mammalian retinas have two types of photoreceptors: rods, which mediate vision at low light intensities, and cones, which operate in bright light and mediate colour vision. The chick retina consists predominantly of cones (Prada et al., 1991). Four different visual pigments are present in chick cones for the preferential absorption of red, green, blue and ultraviolet light (Okano et al., 1995). In addition, the filtering of light by differently coloured oil-droplets is responsible for the further spectral tuning of the cones.

Absorption of light leads to a conformational change in the visual pigment in the outer segment of the photoreceptor. As a result, a cascade of reactions is initiated that lead to hydrolysis of cGMP. In the dark cGMP keeps the ‘light-sensitive’ channels of the outer segment open, while its hydrolysis on exposure to light causes the channels to close. An inward current carried predominantly by Na⁺-ions enters the cell and maintains it in a depolarised state, light closes the outer segment channels and hyperpolarises the cell. This change in membrane potential leads to a reduction of the tonic release of the excitatory amino acid transmitter glutamate from the photoreceptor.
terminal. Both, bipolar and horizontal cells, receive convergent inputs from several cones and respond to the light-induced reduction in glutamate with a graded, sustained potential. Bipolar cells become either depolarised (ON-centre cells) or hyperpolarised (OFF-centre cells) depending on their type. Horizontal cells hyperpolarise in response to light reducing the tonic release of γ-amino-butyric acid (GABA) onto the photoreceptor terminal causing it to depolarise and thus providing the inhibitory surround of those cells in the retina with centre-surround organisation. Bipolar cells, when depolarised, secrete glutamate in turn depolarising ganglion cells and amacrine cells. Ganglion cells respond to light with either an increase or a decrease in the frequency of action potentials they transmit to the brain.

Ganglion cells have been classified on the basis of either their morphology, physiological properties and function or the site of their target cells in the brain. The physiological categorisation begun by Kuffler (1953) divided ganglion cells into ON- and OFF-types based on their receptive field properties. This classification was extended by Enroth-Cugell and Robson (1966), who divided ON and OFF ganglion cells into sustained (X), transient (Y) and ‘other’ types (W) on the basis of their responses to sinusoidal gratings. Wässle et al. (1981) classified cat ganglion cells as α, β and γ cells, the equivalent of Y, X and W ganglion cells, respectively, on the basis of their anatomical properties. In the monkey retina, Kaplan et al. (1986; also Shapley and Perry, 1986) grouped ganglion cells depending on the size of the cells that they contact in the LGN as M (cells projecting to the magnocellular regions of the LGN) and P type (cells projecting to the parvocellular layers of the LGN).
1.2 Development of the retina

1.2.1 Early development

The retina is part of the central nervous system (CNS) located outside the skull. The entire CNS originates from the neural plate, a derivative of the dorsal ectoderm of the gastrula. The plate invaginates along its length to form the neural tube. At the anterior end of the neural tube three primary brain-vesicles emerge, from which the two optic vesicles evaginate (Duke-Elder 1963, Mann 1964). In chick development this occurs at stage 9 (29-33 hrs of incubation) according to Hamburger and Hamilton (1951).

1.2.2 Development of the retinal structure

Once the optic vesicles are fully constricted at their bases, they start to invaginate (stage 14; 50-53 hrs), and within two to three hours the optic cup is completely formed. At this stage it consists of two layers of cells. The outer layer remains one cell thick and forms the pigment epithelium. The cells of the inner layer are neuroblasts that divide repeatedly to produce neurons and glial cells and thus build up the multi-layered neural retina. While the proliferative zone is in the outer part of the neural retina, postmitotic neurons migrate towards the vitread part of the retina to differentiate into ganglion, amacrine and Müller cells. Those with their final destination in the outer part of the retina will become bipolar, horizontal and photoreceptor cells. The somata of the Müller and amacrine cells are separated from the ganglion cell somata by the developing IPL. In a similar way, the OPL forms between the photoreceptor and bipolar and horizontal cell somata.
1.2.3 Cell birth in retinal development

Despite extensive overlap, a class-specific sequence of cell birth is seen in the primate retina: ganglion cells, horizontal cells and cones are born first closely followed by amacrine cells. Bipolar, Müller cells and rod photoreceptors start to be born at the same time as amacrine cells, but their production persists for longer (Rapaport et al., 1996). Similarly, in the developing chick retina ganglion cells are the first cells to leave the cell cycle while bipolar cells are the last (Prada et al., 1991).

Development of ganglion cells

Chick ganglion cells start to be born at E2 and by E3 40% of ganglion cells have already undergone their final mitosis (Snow and Robson, 1994). The cells are at first of bipolar morphology extending one process to the inner limiting membrane and a trailing process that remains in contact with the outer limiting membrane as they migrate towards the vitread part of the retina. At Hamburger-Hamilton's stage 25 (E4) axons extend from the leading process of the cell body and grow towards the optic nerve. Between E5 and E6, the first spiny processes emerge from the cell body and with time a complex dendritic tree develops (Nishimura, 1979). The GCL becomes a distinct layer around E7 and the first synapses are observed in the IPL at E13 before any synaptic specialisations are found in the OPL (Hughes and LaVelle, 1974). This centrifugal (inner to outer) sequence of synaptogenesis in the predominantly cone retina of the chick (Morris and Shorey, 1967; Hughes and LaVelle, 1974) differs from the centripetal pattern of synaptogenesis seen in the predominantly rod retina of rodents (Olney, 1968a and b; Weidmann and Kuwabara, 1969).
1.2.4 Cell death in retinal development

In the developing nervous system more neurons are produced than are actually needed in the adult structure. This excess is removed during development by ‘programmed cell death’ (apoptosis), which occurs at overlapping periods in different cell populations of the retina. In addition, there is innervation- and target-dependent neuronal cell death, which is not a result of lineage and therefore not ‘programmed’, but due to the strengthening effect of correlated synaptic activity either at the level of the dendritic inputs to the neuron or its axonal outputs. Apoptosis occurs in postmitotic as well as in proliferating cells of the developing nervous system (Blaschke et al., 1998).

The chick retina undergoes at least two discrete periods of apoptosis. An early period that coincides with the main onset of neuron birth and migration (E5-E7) (Frade et al., 1997) and a major period of cell death which begins around-E9 and peaks at E10-E11, the time of tectal innervation by ganglion cells (Frade et al., 1997; Cook et al., 1998). Using propidium iodide, which stains pyknotic nuclei (a characteristic of dying cells), the last pyknotic nuclei were seen at E17 (Straznicky and Chehade, 1987). Apoptosis has been observed in the GCL and INL but not in the ONL in retinae between E4 and PD2 (Cook et al., 1998) suggesting that cell death is not a feature common to all the neuron types present. The incorporation of $[^3]H$thymidine at different early stages (E3, E5, E7) shows that cell death does not affect ganglion cells of certain birth dates (Snow and Robson, 1994). While growth and ramification of ganglion cell dendrites between E6 and E10 occurs independent of contact with target cells in the brain, the number and type of surviving cells becomes highly target-dependent during the second half of embryogenesis (Clarke, 1985; Vanselow et al., 1990).
1.3. Amino acid neurotransmitters

There is an abundance of transmitters types found in the CNS including amino acid, monoamine, peptide and purine neurotransmitters. All of these classes of transmitter are present in the retina. As in the CNS, the amino acids glutamate and GABA are present in the retina at concentrations two to three orders of magnitude higher than that of other transmitters.

1.3.1 Release and uptake of amino acid neurotransmitters

a) Glutamate and aspartate

In the human brain intracellular levels of glutamate and aspartate are high (5-10 mM and 2-3 mM, respectively), reflecting the importance of their roles in the metabolism of the cell. Potential pathways for the synthesis of glutamate include transamination and reduction of 2-oxoglutarate by glutamate dehydrogenase or deamination of glutamine by glutaminase. In addition, glutamate can be made from ornithine and proline. The formation of aspartate from oxaloacetate and glutamate is catalysed by aspartate aminotransferase. Synthesis of both amino acids is therefore closely related to the Krebs cycle.

In adult tissue, glutamate is normally released by Ca\(^{2+}\)-dependent exocytosis (deBellecroche and Bradford, 1972; Nicholls, 1989). This vesicular release is triggered by a highly localised increases in [Ca\(^{2+}\)], at release sites (for review see Nicholls and Attwell, 1990). The fact that during persistent action potential activity release occurs in a biphasic temporal pattern suggests that some vesicles are already docked at the release sites and once these have fused with the membrane more vesicles have to be freed from their cytoskeletal attachments and translocated before they can be released. Thus
there is a fast phase of release that occurs within less than a second of depolarisation of the synaptic terminal and a second slower phase.

Once glutamate has been released into the synaptic cleft, Na⁺-glutamate co-transporters scavenge the glutamate from the synaptic cleft to terminate its action. These transporters are distinct from those in the vesicular membrane that depend on ATP for their power (see below). The driving force for the plasma membrane glutamate uptake carrier are the ionic gradients of Na⁺ and K⁺ across the cell membrane: a high concentration of Na⁺ outside and a high concentration of K⁺ inside the cell. Under normal conditions three Na⁺-ions are co-transported with the substrate (glutamate or related compounds) into the cell, while one K⁺-ion and one OH⁻ or HCO₃⁻ are counter-transported (for review see Attwell and Mobbs, 1994), the transport is therefore electrogenic. The plasma membrane carrier, unlike the vesicular transporter, will also transport aspartate and can be inhibited by kainate.

Five homologous types of Na⁺/K⁺-dependent high affinity glutamate transporters, EAAT1-5, have been cloned (for review see Gegelashvili and Shousboe, 1997). While EAAT1 (GLAST) and EAAT2 (GLT-1) are found in glial cells EAAT3 (EAAC1) to EAAT5 are localised in the dendrites and somata of CNS neurons (EAAT5 is found only in the retina). The glutamate taken up by neurons is pumped back into vesicles by the vesicular transporter, while glutamate taken up by glia is converted into glutamine by glutamine synthetase which is present only in glial cells. Glutamine is free to diffuse back into neurons to replenish the pool of glutamate following hydrolysis in the mitochondria.

Glutamate uptake into vesicles is Na⁺-independent and is driven by the proton gradient across the vesicle membrane generated by the inwardly directed vesicular proton-ATPase (for review see Ozkan and Ueda, 1998). This carrier has a low affinity for glutamate (in the mM range) and does not transport aspartate. The role of aspartate as a neurotransmitter is not clear.
Until recently it was believed that aspartate, like taurine, is neither stored in vesicles nor released by exocytosis (for review see Fykse and Fonnum, 1996), which may explain why the release of these amino acids from synaptosomes is Ca\textsuperscript{2+}-independent.

During brain ischemia neurons undergo long-lasting depolarisation that leads to major changes in ionic concentrations, that can cause the glutamate uptake carrier to run backwards (Szatkowski et al., 1990; Madl and Burgesser, 1993). Reversed uptake can raise extracellular glutamate concentrations to levels known to trigger neuronal cell death (for review see Szatkowsky and Attwell, 1994).

b) GABA and glycine

GABA is synthesised by decarboxylation of glutamate, a reaction that is catalysed by glutamate decarboxylase (GAD) through a shunt of the Krebs cycle. Glycine is synthesised by transamination of serine, which is derived from 3-phosphoglycerate and through this linked to glycolosis.

The release of GABA and glycine at most synapses in the retina is via Ca\textsuperscript{2+}-dependent exocytosis. However, there is some evidence that glycine induced release of GABA from horizontal cells occurs via a Ca\textsuperscript{2+} independent mechanism (Smiley and Basinger, 1990), which may involve reversed uptake. It has been suggested that, early in development, reversed uptake is a major mechanism for the release of GABA and possibly also glutamate (Taylor and Gordon-Weeks, 1991).

Uptake of GABA into synaptic vesicles is via a GABA/H\textsuperscript{+} exchange carrier. The action of GABA in the synaptic cleft is terminated by diffusion and uptake into synaptic terminals or glia. Four distinct GABA transporters have been cloned (GAT1-3 and BGT1), all of them are found in CNS neurons and glia.
(Itouji et al., 1996; Johnson et al., 1996; Borden et al., 1995; Minelli et al., 1995). These uptake carriers are driven by the Na$^+$-gradient and co-transport 1 Na$^+$ and 1 Cl$^-$ with the GABA-ion, GABA uptake is therefore electrogenic. Patch clamp studies have shown that GABA transporters also need Cl$^-$-ions or alkali metal ions inside the cell to operate (Cammack and Schwartz, 1993). Uptake carriers for glycine, dopamine, serotonin and noradrenaline belong to the same family of transporters. Two glycine transporters (GLYT1, 2) have been identified, one of which (GLYT1) is glial (Zafra et al., 1995) and the other (GLYT2) neuronal (Araki et al., 1988; van den Pol and Gorcs, 1988; Wenthold et al., 1988; Jursky and Nelson 1995). In the retina though, GLYT1 has been localised in amacrine cells (Zafra et al., 1997).

1.4 Amino acid neurotransmitter receptors

1.4.1 Glutamate and aspartate

Glutamate is the primary mediator of excitatory transmission in the CNS and the retina, and plays key roles in neuronal development. The major endogenous activators of excitatory amino acid receptors are L-glutamate and L-aspartate. However, cysteine as well as other sulfur-containing derivatives and quinolinic acid are also endogenous agonists. The pharmacology of these receptors has revealed at least five groups of glutamate receptors in the vertebrate CNS (for review see Ozawa et al., 1998 and Michaelis, 1998). Three of these are linked directly to ion channels (ionotropic) and have been classified into N-methyl-D-aspartate (NMDA)- and non-NMDA receptors (for review see Nakanishi et al., 1998; Fletcher and Lodge, 1996). The latter group is divided on the basis of activation by the selective agonists, (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (L-AMPA) and kainate, into AMPA-prefering receptors, with a much higher affinity for AMPA, and kainate-prefering receptors, with a higher affinity for kainate (Monaghan et al., 1989). In this thesis these will be
referred to as AMPA and kainate receptors, respectively. The expression cloning of cDNA's for different subunits of the ionotropic glutamate receptor have demonstrated that GluRA to D (GluR1 to 4) form channels with a high affinity for AMPA and glutamate and a low affinity for kainate (here termed the AMPA receptor), while GluR5 to 7 and KA1 and 2 form receptors with a high affinity for kainate (kainate receptors). Receptors formed from subunits termed NMDAR1 and NMDAR2 (A-D) are activated by glutamate and NMDA, but not by AMPA or kainate (NMDA receptors). The other two groups of glutamate receptors are coupled to G-proteins (metabotropic receptors). Quisqualate can activate both, some ionotropic and a group of metabotropic glutamate receptors that are selectively activated by [1S3R]-1-amino-1,3-cyclopentanedicarboxylic acid (trans-ACPD). In addition, a second type of metabotropic glutamate receptor can be identified using L-2-amino-4-phosphonobutanoic acid (AP4). The AP4 receptor seems to be an autoreceptor whose function is to inhibit glutamate release. Different subtypes of glutamate receptors can coexist in one cell (Michaelis, 1998).

a) Non-NMDA receptors

The first selective agonist used to separate non-NMDA from NMDA receptors was quisqualate, a plant toxin from *Quisqualis indica*. However, quisqualate was found to activate metabotropic receptors and AMPA turned out to be a more selective and useful agonist. The non-NMDA receptors activated by AMPA in preference to kainate are simply termed AMPA receptors. The synthesis of 6-nitro-7-cyanoquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 2,3 dihydroxy-6-nitro-7-sulfamoylbenzo(F)-quinoxaline-2,3-dione (NBQX), which have little affinity for the glycine site of the NMDA receptor and are potent and selective for non-NMDA antagonists, have enabled extensive characterisation of non-NMDA receptor function.
AMPAn receptors

AMPAn receptors mediate fast excitatory transmission. Their activation is characterised by a rapidly activating current followed by nearly complete desensitisation (Trussell and Fischbach, 1989; Jonas and Sakmann, 1992; Colquhoun et al., 1992). In contrast, their activation by kainate leads to currents that are maintained as long as the drug is present (Keinanen et al., 1990; Patneau and Mayer, 1990). In general, the ion channels linked to AMPAn receptors are permeable to Na\(^+\) and K\(^+\), but allow only little influx of Ca\(^{2+}\)-ions due to the presence of the GLUR2 subunit.

Kainate receptors

Application of kainate causes a transient response at kainate receptor-coupled ion channels (Huettner, 1990), which is unlike the sustained response seen at AMPAn receptors. Lectins, such as concavalin A, greatly potentiate kainate responses, but are a lot less affective at AMPA/kainate receptors (Partin et al., 1993; Wong and Mayer, 1993). Like the AMPA/kainate receptors a cation channel is linked to the kainate receptor that is far more permeable to Na\(^+\)-and K\(^+\)- than to Ca\(^{2+}\)-ions and is blocked by CNQX and related compounds.

b) NMDA receptors

Unlike non-NMDA receptors, the NMDA receptor is not believed to function as a mediator of fast synaptic transmission, because they respond more slowly and their contribution is primarily to the slow component of excitatory postsynaptic currents (Forsythe and Westbrook, 1988; Trussel and Fischbach, 1989; Gibb and Colquhoun, 1992). The slow, long-lasting response of these receptors is thought to be due to the high affinity of the
receptor to glutamate (for review see Zorumski and Thio, 1992). In addition, the responses of NMDA receptors to glutamate show a voltage-sensitivity that is due to a voltage-dependent block of the channel by Mg$^{2+}$-ions in the extracellular medium (Mayer et al., 1984; Nowak et al., 1984). Other divalent cations such as Ni$^{2+}$, Co$^{2+}$ and Mn$^{2+}$ are also antagonists of NMDA receptor activated ion channels. The block by these ions is voltage-dependent and can be relieved by depolarisation. In 1987, Johnson and Ascher discovered that glycine binding is necessary for glutamate to activate NMDA receptor channels. The glycine binding site on the NMDA receptor is not blocked by strychnine as it is for glycine receptor. Kynurenic acid, an NMDA receptor antagonist works by displacing glycine from its binding site on the receptor (Kemp et al., 1988).

NMDA receptors are selectively antagonised by 2-amino-5-phosphonovaleric acid (AP5), 2-amino-7-phosphonoheptanoic acid (AP7) and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CCP) (for review see De Sarro et al., 1995). These are competitive antagonists that bind to the glutamate NMDA binding site without activating the ion channel. Non-competitive antagonists like PCP bind to a site within the ion channel of the NMDA receptor complex. MK-801 is another high affinity ligand for the PCP receptor site, which was originally produced by Merck for use as a possible neuroprotectant. The NMDA receptor differs from other glutamate receptors in its sensitivity to pH (Monaghan and Cotman, 1986; Traynelis and Cull-Candy, 1990) and voltage-independent blockade by Zn$^{2+}$ (Westbrook and Mayer, 1987). The NMDA receptor channel is highly permeable to Ca$^{2+}$ (Monyer et al., 1992), a feature that is believed to play an important role in synaptic plasticity.
Metabotropic glutamate receptors produce the most widespread physiological and biochemical effects on neurons of any of the glutamate receptors. They can be divided into three groups (I-III) (for review see Michaelis, 1997). Group I receptors are coupled either to pertussis toxin sensitive or insensitive G-proteins. Activation of the receptor leads to the phospholipase C (PLC)-catalysed conversion of L-3-phosphatidylmyoinositol-4,5-bisphosphate (PIP$_2$) to inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) (Sladeczeck et al., 1985; Nicoletti et al., 1986; Novelli et al., 1987). The latter is involved in the activation of protein kinase C (PKC), while IP$_3$ causes release of intracellular Ca$^{2+}$ from the endoplasmatic reticulum (for review see Berridge and Irvine, 1984). Activation of Group II receptors causes stimulation of adenyl cyclases, which appears not to be directly coupled to G$_s$-protein (for review see Schoepp and Conn, 1993). Group III receptor activation leads to the opposite effect in that it inhibits adenyl cyclases through direct interaction with G$_i$-proteins (Prezeau et al., 1992). All of these receptors are activated by glutamate and 1-aminocyclopentane-1s,3R-dicarboxylic acid (1s,3R-ACPD). Their sensitivity to quisqualate, ibotenate, 2-(carboxycyclopropyl)glycine (L-CCG) and L-2-amino-4-phosphonobutanoic acid (2-AP4) varies within these groups depending on cell type (Prezeau et al., 1994) suggesting considerable variability in the configuration of metabotropic glutamate receptors expressed in different neuronal populations of the CNS.

The cloning of the cDNA for metabotropic receptors has led thus far to the identification of 8 different forms; mGluR1-8, with mGluR1-3 and 5 are activated by trans-ACPD, while mGluR4 and 6-8 can be activated by L-AP4 (Nakanishi, 1992; Nakajima et al., 1993; Okamoto et al., 1994; Saugstad et al., 1994; Duvoisin et al., 1995)
1.4.2 GABA and glycine

GABA is the major inhibitory transmitter of the adult CNS and visual system. It mediates fast synaptic inhibition through activation of a Cl⁻ channel that leads to hyperpolarisation of the cell (Bormann 1988; Feigenspan et al. 1993). During development, the action of GABA on immature neurons is often depolarising (Segal and Barker 1984; Cherubini et al., 1991). The shift from depolarising to hyperpolarising responses to GABA with time reflects a change in the Cl⁻ equilibrium potential (Owens et al., 1996). Glycine plays an important role in the brain stem, spinal cord and INL/IPL of the retina analogous to that played by GABA in the rest of the CNS. In addition to its inhibitory effect in these regions it acts as a co-activator of the NMDA receptor throughout the CNS. GABA and glycine receptors are closely homologous and below are considered together.

There are at least three pharmacologically distinct GABA receptor subtypes: GABAₐ, GABA₉, GABAₐ (for review see Silvilotti and Nistri, 1991). GABAₐ receptors are selectively activated by muscimol and selectively blocked by bicuculline and picrotoxin. Baclofen is a specific agonist, and CGP55845A a specific antagonist, of GABA₉ receptors. While the GABAₐ receptor complex contains an integral chloride channel, GABA₉ receptors are indirectly coupled to both calcium and potassium channels via G-protein activation (reviewed by Bormann, 1988 and Bowery, 1993). The GABAₐ receptor contains a binding site for the benzodiazepines. Benzodiazepines (BZD’s) are anxiolytic, antiepileptic and muscle relaxant drugs, that potentiate the inhibitory effect of GABA by increasing the channel opening frequency and thus increasing the Cl⁻ current. The third receptor subtype, the GABA₉ receptor, is insensitive to bicuculline and baclofen and can be activated by cis-4-aminocrotonic acid. These receptors are also linked to a Cl⁻-channel, but differ from the GABAₐ receptor in their lack of modulation by BZD and barbiturate (Cutting et al., 1991; Shimada et al., 1992). The molecular biology
of GABA and glycine receptors is complex (see for review Wässle et al., 1998) and outside the scope of this introduction.

1.5 Gap junctions in the retina

Most cells communicate with their neighbours through the exchange of ions, second messengers and small metabolites. The transfer of these between cells is made possible by clusters of intercellular channels called gap junctions. Gap junctions mediate both metabolic and electrical communication. The following section reviews the structure and function of gap junctions with particular reference to their roles in retinal development.

1.5.1 Historical Perspective

Gap junctions were first observed in neuronal cells in an electron microscopical (EM) study by Robertson (1963) in the club endings of Mauthner neurons in the goldfish brain. Two years later, Rosenbluth (1965) described their basic anatomical characteristics and differentiated them from tight junctions. However, the first extensive study of gap junctions and the one which gave them their current name was carried out by Revel and Karnovsky in 1967. The newly developed colloidal lanthanum negative staining method and uranyl acetate en bloc staining enabled them to relate the hexagonal arrays seen in tangential sections to the junctional membranes displaying a fixed 2nm gap. The first pictures of freeze fracture replicas of gap junctions were published by Kreutziger (1968), who demonstrated that the intramembranous particles seen in replicas corresponded to the structures seen as the hexagonal lattices in conventional EM sections.
1.5.2 The structure of gap junctions

A gap junction is an aggregation of membrane proteins arranged in hexagonal arrays in each of two adjoining membranes. These proteins are attached in such a way as to fix a gap of 2-3nm between the two membranes.

In EM thin sections, gap junctions appear as heptalaminar structures between apposed membranes with a fixed gap of 2-3 nm. In freeze fracture replicas, they appear as intramembranous particles and complementary pits that form a complex of 8-9 nm in diameter. We now know that gap junctions are clusters of plasma membrane channels that directly link the cytoplasm of two cells. Each gap junctional channel is of ~ 2.5 nm in length and ~ 1.5 nm in diameter and projects across the gap of 2-3 nm (for review see Bennett et al., 1991). It is formed by two hemi-channels, called connexons, each spanning the full width of one junctional membrane. Connexons are made up of six subunits (for review see Peracchia, 1980) each of which are single integral membrane proteins termed 'connexins' (for review see Severs, 1995).

Connexins

At the present time, 13 different connexins have been described in rodents which are principally distinguished by their molecular mass (KDa) (for review see Beyer et al., 1990). They comprise a multigene family of two distinct lineages: class 1 or b (eg. Cxs 26, 31, 32) and class 2 or a (e.g. 37, 40, 43, 45). Their gene structures exhibit 50% sequence homology (for review see Dermietzel and Spray, 1993).
A) Figure 1.2

B) A schematic diagram of a gap junctional channel.

B) Gap junctional channels become functional when two hemi-channels, which are called connexons (green) and which are located in apposing plasma membranes (red), ‘dock’ together. Each connexon consists of six gap junctional proteins, called connexins. (Taken and modified from ‘Molecular Biology of the Cell’, 3rd edition).

Lower panel: A schematic diagram of a connexin molecule. Each connexin consists of four transmembrane domains, two extracellular and one intracellular loop. The carboxy- and the amino-terminus of the protein are both located in the cytoplasm. The green bands show the location of the peptides to which the Cx 32 antibodies that were used in the experiments of Chapter 5, were raised. And the red bands show the location for the antibodies raised against Cx 43. (Taken from K. Davies).
Two connexons 'dock' together to form a channel between cells

Modified from Molecular Biology of the Cell, 3rd Ed.
A connexin molecule contains four transmembrane domains with cytoplasmic amino and carboxyl termini (for review see Beyer et al. 1990). These proteins are highly conserved in their extracellular and transmembrane domains, but are structurally diverse in their cytoplasmic termini, variation which may account for their different properties (Zimmer et al., 1987, Laird and Revel, 1990). The fact that the extracellular domains contain three cysteine residues located at identical positions (Rahman and Evans, 1991) suggests that they may be important in the docking mechanism of the two hemi-channels and/or in channel opening (Dahl et al., 1992). However, part of the protein that controls the selection of the apposing connexin has been shown to lie in the second extracellular domain (White et al., 1995).

Specific connexins exist in more than one tissue and a single cell can express more than one type. Communicating channels can be formed by identical hemi-channels (homotypic gap junction) or by hemi-channels comprising different connexin types (heterotypic gap junctions). Transfection of human HeLa cells with mouse DNA coding for Cx 26 and Cx 32 reveal its ability to form heterotypic channels with biophysical properties different to those of the homotypic channels (Bukauskas et al., 1995). However, this is not true of all Cxs, for example murine Cx 40 and Cx 43 expressed in HeLa cells do not form functional channels (Haubrich et al., 1996). Experiments with chimeric constructs, with different combinations of extra- and intracellular domains of both Cxs suggest that the ability of Cxs to associate as functional channels is determined by the nature of their intracellular domains. Expression of rodent lens Cxs in paired Xenopus oocytes has shown that the second extracellular domain may also play a role in determining the compatibility of Cxs (White et al., 1995).
1.5.3 Synthesis, assembly and degradation of connexons

As with most integral membrane proteins, connexins are co-translationally inserted in the rough endoplasmic reticulum (Falk et al., 1994), but will not oligomerize before transported into the trans-Golgi compartment (Musil and Goodenough, 1993). This delayed assembly of the connexins in the vesicles of the trans-Golgi may prevent intra-reticular channel formation. Overexpression of connexins in transfected cultured cells results in 'inappropriate' intracellular channels between the apposed membranes of the cisternae of the ER and nuclear envelope (Kumar et al., 1995). Connexons are transported to the plasma membrane and, in physiological conditions, remain closed until a complete intercellular channel is formed. One study by Musil and Goodenough (1991) has demonstrated that connexins are transported to the cell surface in an unphosphorylated form. Another study has shown that connexins undergo post-translational modifications through phosphorylation, a cAMP-dependent mechanism that promotes the translocation of Cx 43 into gap junctional plaques; thus increasing intercellular communication (Atkinson et al., 1995).

Connexins are dynamic proteins. The rapid formation of gap junction channels (within seconds to minutes) suggests the pre-existence of hemi-channels in the membrane (Rook et al. 1990), while a short half-life of 1.5 to 5 hours (in cultured cells and living animals) indicates that there is a high turn-over of these proteins. Degradation appears to involve ubiquitin-mediated proteolysis (Laing and Beyer, 1995; Bruzzone et al., 1996).

1.5.4 Regulation of gap junctions

Gap junctions vary in their conductances depending on the type of connexin that forms the hemi-channels (Moreno et al., 1992; Takens-Kwak and Jongsma, 1992). The nature of the junction determines the characteristics of
cell-to-cell communication. Gap junctions are plastic structures and are modulated through the action of neurotransmitters, changes in intracellular [pH], the transmembrane voltage gradient, second messengers (f.e. cAMP, cGMP, Ca\textsuperscript{2+} and IP\textsubscript{3}) and other factors (for review see Bennett et al, 1991 and see Rörig and Sutor, 1996; and see below).

a) Regulation by dopamine

Teranishi et al. (1983) showed using intracellular injections of Lucifer yellow that dopamine reduces the gap junctional coupling between the horizontal cells of the carp (Cyprinus carpio). They also showed that perfusion of the isolated retina with the GABA antagonist bicuculline (20 μM) had the same effect and was mediated via the release of dopamine. This was demonstrated by killing the dopaminergic cells in the retina with 6-hydroxydopamine prior to the application of GABA. In retinae treated this way, GABA did not produce an uncoupling effect. Dopamine also reduces the coupling between rabbit All amacrine cells (Hampson et al., 1992), however, the effects of dopamine in the retina are not universal since it has no effect on the gap junctions between All amacrine cells and bipolar cells (Mills and Massey, 1995).

The decrease in coupling between horizontal cells produced by dopamine is interpreted as a loss of gap junctions since freeze-fracture replicas of dopamine treated goldfish retinae show a lower gap junction particle density in their membranes (Baldrige et al., 1987; 1989). The same reduction was observed in light-adapted retinae suggesting that there is a greater release of dopamine under photopic than scotopic conditions. By voltage-clamping pairs of teleost horizontal cells, direct measurements of the conductance between cells suggests a unitary channel conductance of 50-60 pS (McHahon et al., 1989). McHahon et al. suggest that dopamine reduces the open probability of gap junctional channels by decreasing their open duration. The action of dopamine on horizontal cells is mediated by the
intracellular second messenger cAMP via D₁ receptors, an increase in cAMP activates a cAMP dependent protein kinase inducing phosphorylation of the gap junction proteins (Piccolino et al., 1984; Lasater, 1987).

Binding studies of retinal dopamine receptors in adult chick retinas have revealed the existence of both D₁- and D₂-subtypes (for review see Schorderet and Nowak, 1990). In the embryonic chick retina D₁-receptors were found in the IPL and OPL as well as in the INL (Elena et al., 1989). In the adult rat retina D₁-receptors were localised on horizontal and cone bipolar cells and a small number of amacrine cells, but there was no co-localisation of D₁-receptors with tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines (Veruki and Wässle 1996). The spatial disparity between dopaminergic processes and the site of the D₁-receptors is consistent with the notion that in the retina dopamine acts as a signal that diffuses through the extracellular space. In addition, de Mello et al. (1996) have distinguished an atypical D₁-receptor subtype which is transiently expressed in cultures of the embryonic chick retina. This atypical receptor, unlike its counterpart in the adult retina, irreversibly binds to SCH 23390, a D₁-receptor family blocker. D₂-subtype receptors may be present in cultured photoreceptors, where luvone et al. (1990) have shown that dopamine suppresses cAMP synthesis and thus inhibits serotonin N-acetyltransferase (NAT) activity. This action was blocked by D₂, but not by D₁ receptor antagonists. The majority of dopamine receptors in the retina are coupled positively to adenylate cyclase activity indicating that they are of the D₁-subtype (for review see Schorderet and Nowak, 1990).

The dopaminergic cells in the retina can be revealed using immunohistochemical techniques for tyrosine hydroxylase, the rate limiting enzyme in the catecholamine synthetic pathway. Such staining methods show two different types of amacrine cells to be dopaminergic, one is of the interplexiform cell type and the other was located in the INL, IPL and GCL and has thick monostratified dendrites (Crooks and Kolb, 1992). The
dopaminergic interplexiform cells form synapses mostly with other amacrine subtypes, but also with other interplexiform cells, bipolar cell terminals and unidentified processes and cell bodies in the IPL, GCL and AFL (Yazulla and Zucker, 1988). Thus, the input to dopaminergic cells, which are mainly interplexiform cells, comes from bipolar and other amacrine cells.

b) Regulation through changes in pH,

In the adult neocortex, gap junctional coupling between astrocytes is highly sensitive to cytoplasmic acidification, while neuronal coupling is relatively insensitive (Conners et al., 1983). Kettenmann et al. (1990) using cultured mouse oligodendrocytes, showed that lowering the pH to less than 6.5 caused a transient block of electrical coupling between these cells. In the developing neocortex coupling between immature pyramidal cells is reduced by 64% following a pre-incubation of the tissue in sodium salts of weak organic acids (eg. Na\(^+\)-propionate) (Rõrig et al. 1996). To prove that the change in the electrical coupling of these cells was due to the uncoupling effect of the acid, the electrical properties were compared in undifferentiated and differentiated cells in the presence of Na\(^+\)-propionate. These neurons become uncoupled as differentiation proceeds and Na\(^+\)-propionate was without effect at these later times suggesting the uncoupling seen at early times was indeed due to an action of this weak acid at gap junctions.

In the adult retina, coupling between horizontal cells is also dependent on the extracellular pH; the cells uncouple below pH 7.0 (Hampson et al. 1992). Given that the pH in the outer retina varies with the metabolic activity of the photoreceptors, Hampson et al. suggest horizontal cell coupling may be modulated in the intact retina via a pH sensitive mechanism.
c) Regulation by nitric oxide (NO)

In the adult mammalian retina gap junctions between All amacrine cells and bipolar cells are not effected by dopamine and the cAMP cascade, but rather are modulated by NO and cGMP (Mills and Massey 1995). Since All amacrine cells integrate rod signals into the bipolar pathway via gap junctions, this result suggests that NO turns the switch between rod and cone vision as light intensities increase.

In the developing rat sensorimotor cortex, gap junctional conductance is controlled by the NO/cGMP system as well. Incubation in sodium nitroprusside, a NO donor, leads to a decrease in the number of coupled cells shown using injections of neurobiotin (Rörig and Sutor, 1996b).

d) Regulation by serotonin

The gap junctional coupling between developing neurons in the neocortex is not only sensitive to NO but also to serotonin, which reduces dye-coupling through its action on 5-HT receptors. Heparin as well as the protein kinase C inhibitor NPC 15437 opposes this effect suggesting that it involves IP₃ receptor-mediated release of Ca²⁺ ions from intracellular stores (Rörig and Sutor 1996a). Thus, it appears that serotonin regulates some gap junctional conductances in development. The importance of such regulatory mechanisms is not understood.

e) Regulation by other substances

The effect of hormones and cAMP on the modulation of gap junctional coupling between non-neuronal cells and in cell lines has been studied extensively. Findlay and Petersen (1982) were first to demonstrate hormonal
effects on gap junction permeability showing an increased coupling between pancreatic acinar cells with application of acetylcholine. A similar effect was found in osteoblast cell lines exposed to parathyroid hormone (PTH) for one hour where coupling increased in a dose-dependent manner and involved an increase in cAMP (Donahue et al., 1995). Direct incubation of rat myocytes cell cultures in dibuturyl-cAMP increased the number and size of gap junctions and the amount of both Cx 43 and Cx 45 (Darrow et al., 1996). While Darrow et al. found an increased level of Cx 43 mRNA, this was not true for Cx45 mRNA suggesting that Cx 45 protein levels were raised by a higher rate of synthesis rather than an increase in mRNA. Thus, in these cells Cx 43 and Cx 45 levels appear to be upregulated by cAMP through different molecular mechanisms.

1.5.5 Gap junctions in the adult vertebrate retina

In the adult retina there are gap junctions between almost all cell types but not between horizontal and bipolar cells, ganglion and bipolar cells or ganglion cells and photoreceptors (for review see Cook and Becker, 1995). The physiological role of many of these junctions is poorly understood. However, it is well known that gap junctions between horizontal cells in the OPL and between amacrine cells in the IPL form a substrate for the modulation of the receptive field size and properties of bipolar cells and ganglion cells.

a) Photoreceptors

In all vertebrates that have been studied so far, the photoreceptors are coupled by gap junctions. There is homotypic coupling of rod to rod and cone to cone (Baylor et al., 1971; Raviola and Gilula, 1973; Schwartz, 1976), and heterotypic coupling between rod spherules and cone pedicles (Scholes,
1975; Copenhagen and Owen, 1976). The extent of coupling of cone pedicles is high: in the area centralis of the cat each is coupled on average to 48 rod spherules (Smith et al., 1986). In non-mammals, coupling tends to occur between cone receptors with the same spectral properties (Detwiler and Hodgkin, 1979; Gold and Dowling, 1979).

The functional importance of photoreceptor coupling is that it reduces the noise by pooling the responses of neighbouring cells (Tessier-Lavigne and Attwell, 1988; see for review McNaughton, 1990); coupling maximises sensitivity to small light spots, albeit with a concomitant loss of spatial discrimination; and in the case of heterotypic rod to cone coupling, provides a pathway by which the visual signal passes into the centre-surround-system at mesopic light levels.

b) Horizontal cells

Horizontal cells are strongly coupled forming type-specific syncytial networks (Raviola and Gilula, 1975), and their receptive fields are thus much larger than their dendritic fields (Bloomfield and Miller, 1982; Dacheux and Raviola, 1982). Axon-bearing types of mammalian horizontal cells were at first thought not to be coupled, but Vaney (1994) has demonstrated using Neurobiotin that they are dye-coupled. The site of coupling is between dendrites and between terminal arbors.

Horizontal cells feed graded, low-noise inhibitory signals back to the photoreceptors in order to moderate the high gain of the receptor cell to make best possible use of its dynamic range (Jacobs and Werblin, 1998). In addition, in many vertebrates specialised horizontal cell subtypes, termed A-C, are responsible for colour opponency and inhibit cones of one spectral class when those of another are illuminated, a role similar to that of contrast enhancement (for review see Wu, 1992; Dacey, 1996).
c) Bipolar cells

Coupling occurs between bipolar of the same subtype (ON- and OFF-centre cells) and between ON-centre bipolar and All amacrine cells (Saito and Kujiraoka 1988; Cohen and Sterling 1990). Dye-injections show the sites of coupling to lie between dendrites (carp retina, Saito and Kujiraoka 1988) or axon terminals (elasmobranch fish, teleost fish, amphibians, mammals) and telodendria (reviewed by Marc et al. 1988; Vaney 1994).

d) Amacrine cells

Naka and Christensen (1981) have shown using transmission EM of horseradish peroxidase (HRP) stained retinae that both chemical synapses and gap junctions connect amacrine cells of the same anatomical class. There is strong evidence in the cyprinid retina that at least four amacrine subtypes form homotypic mosaics through gap junctions (Hidaka et al., 1993). All amacrine cells, the most studied type of amacrine cell, are more highly coupled in dim light than in complete darkness as are horizontal cells (Bloomfield et al., 1997). But, unlike horizontal cells, most amacrine cell subtypes show no signs of receptive field enlargement in electrophysiological recordings even when they are extensively tracer-coupled (Bloomfield, 1992; Bloomfield and Xin, 1997). All-type coupling is reduced by activation of D_1-receptors, but not by changes in pH_ex and therefore presumably not affected by pH_i (Hampson et al., 1992).

e) Ganglion cells

In the cat α-ganglion cells are coupled to form independent inner- and outer-stratified mosaics (for review see Vaney 1991; Wässle et al. 1981), while cat β cells, like monkey midget ganglion cells, are not tracer-coupled (see Vaney,
1991; Dacey and Brace, 1992). Penn et al. (1994) has shown homotypic coupling between not only the α-, but also γ-ganglion cells in the ferret retina. Heterotypic coupling to amacrine cells of one or more classes has also been reported (Dacey and Brace, 1992; Penn et al., 1994).

f) Glial cells

In amphibian (Axolotl) retinas Müller cells have been shown to be highly coupled in electrophysiological and dye-transfer experiments (Mobbs et al., 1988). In the mammalian retina there is extensive coupling between glial cells (Robinson et al. 1993). Injections of Neurobiotin reveal that astrocytes are dye-coupled to other astrocytes and, in addition, to Müller cells (Zahs and Newman, 1997). These gap junctions turn out to be ‘asymmetric’, in that dye-transfer into Müller cells followed from dye-injections into astrocytes but not vice versa.

1.5.6 Gap junctions in development

Gap junctions are thought to play a key role in intercellular communication during development. In the moulting moth Manduca sexta f.e. transient gap junctional pathways are established in a small temporal window before each moult (Baldwin et al., 1993). And injection of antibodies for different connexins into the amphibian embryo has shown severe disruption of the normal pattern formation in the non-communication area (Warner, 1985). This section briefly reviews the coupling seen between neuroblasts and newly formed neurons of the embryonic nervous system.

In the developing brain, there is extensive dye-coupling (Lucifer yellow) between the neuroblasts of the cortex. Neurobiotin injections show transient coupling between neurons in the post-migratory phase but before
synaptogenesis begins (Peinado et al., 1993). It has been suggested that the spontaneous activity seen in the cortex is caused by the activity of a few trigger cells which propagates via gap junctions through the remaining cells that comprise a domain (Yuste et al., 1995).

In early development of the embryonic chick retina gap junctions are evident at E3 in the outer part of the retina in thin sections and freeze-cleaved material (Fujisawa et al., 1976). Fujisawa found a steep increase in the number and size of gap junctions at E5 and a sudden drop between E7.5 and E9. These results are supported by the study of Sheffield (1980), who found a sudden drop in gap junctions between cells at the outer limiting membrane at E10. At early stages gap junctions exist among pigment epithelial cells, among ventricular cells and between these two cell types (Hayes, 1977), but with time the heterotypic junctions between neuronal and epithelial cells disappear. The presence of gap junctions between ganglion cells and between ganglion cells and amacrine cells in the ferret retina at times when firing of retinal ganglion cells is highly correlated (Penn et al., 1994) suggest a role in synchronisation of the spontaneous activity seen in the ferret retina.

The importance of gap junctions in neuronal development can be demonstrated by using antisense techniques to particular Cxs. With this method the normal pattern of intercellular communication is disrupted and thus developmental mechanisms are drastically perturbed (for review see Guthrie and Gilula, 1989). Mutant mice without Cx 32 have abnormal myelin sheaths (Anzini et al. 1997), indicating an important role of this protein in myelination during embryonic development.

The aim of this thesis is to investigate intercellular communication during early retinal development, which can be mediated chemically and electrically by amino acid transmitters and gap junctions.
Chapter 2

Methods

2.1 Preparation of the retina

The chick embryos used in the course of this thesis were of the strain White-Leghorn. Fertile eggs were kept in a rocking incubator at 38°C with humidified air. Animals from embryonic day 5 to 16 (E5-16) were sacrificed by decapitation according to Schedule 1 of the Home Office Regulations (1986). The whole eyeball was removed from the head and adherent muscle tissue was trimmed away and a cut was made around the eye at the level of the ora serrata. After discarding the cornea and iris, the sclera with the pigment epithelium and the vitreous humor could be removed using watchmaker forceps. For all experiments, except for the measurements of amino acid transmitter release, a small square was cut from the centre of the retina just above the choroid. This area is known to be the most mature region of the developing retina. Using the blunt end of a pasteur pipette the tissue was transferred to oxygenated HEPES-buffered Avian Ringer’s solution (Solution B) or bicarbonate-buffered Krebs’ solution (Solution C) for further treatment.

2.2 Solutions

The composition of the various solutions used are shown in the tables below.
Table 2.1: Intracellular solution

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Intracellular medium</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>40.00</td>
<td></td>
</tr>
<tr>
<td>NMDG¹-EGTA</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Cs-gluconate</td>
<td>103.00</td>
<td></td>
</tr>
<tr>
<td>Na₂ATP</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.00²</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2: Extracellular solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avian Ringer's solution</td>
<td>Control</td>
<td>High K⁺</td>
<td>Control/ high Mg²⁺</td>
<td>High K⁺/ high Mg²⁺</td>
</tr>
<tr>
<td>NaCl</td>
<td>136.90</td>
<td>100.00</td>
<td>40.00</td>
<td>100.00</td>
<td>40.00</td>
</tr>
<tr>
<td>KCl</td>
<td>5.30</td>
<td>6.00</td>
<td>60.00</td>
<td>6.00</td>
<td>60.00</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.41</td>
<td>1.00</td>
<td>1.00</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>3.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>HEPES</td>
<td>3.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>30.00</td>
<td>30.00</td>
<td>30.00</td>
<td>30.00</td>
<td>30.00</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>glucose</td>
<td>5.60</td>
<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>pH</td>
<td>7.40³</td>
<td>7.36⁴</td>
<td>7.36⁴</td>
<td>7.36⁴</td>
<td>7.36⁴</td>
</tr>
</tbody>
</table>

¹ NMDG is N-methyl-D-glucamine; EGTA is ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
² with HCL
³ with NaOH
⁴ gassed with 95% O₂/ 5% CO₂
Table 2.3: HPLC Buffers

<table>
<thead>
<tr>
<th>Solution</th>
<th>Buffer A</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>72%</td>
<td>42%</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>8%</td>
<td>30%</td>
</tr>
<tr>
<td>methanol</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td>Na-propionate</td>
<td>20%</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: OPA-solution

<table>
<thead>
<tr>
<th>OPA-solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
</tr>
<tr>
<td>Na-borate$^5$</td>
</tr>
<tr>
<td>mercaptoethanol</td>
</tr>
<tr>
<td>o-phthalaldehyde (OPA)</td>
</tr>
<tr>
<td>pH</td>
</tr>
</tbody>
</table>

Table 2.5: Tris- Buffer

<table>
<thead>
<tr>
<th>Solution G</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA/Tris with 1% SDS</td>
</tr>
<tr>
<td>EDTA$^6$</td>
</tr>
<tr>
<td>Tris$^7$</td>
</tr>
<tr>
<td>SDS$^8$</td>
</tr>
</tbody>
</table>

$^5$ stock solution is 1 M and pH 10.4
$^6$ Ethylenediaminetetraacetic acid
$^7$ Tris(hydroxymethyl)methylamine
$^8$ Na-duodecasulphate
Table 2.6: HPLC Amino Acid Standard Solutions

<table>
<thead>
<tr>
<th>Amino Acid Standard Solution</th>
<th>&quot;Low Standard&quot; pmoles</th>
<th>&quot;High Standard&quot; pmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartate</td>
<td>33</td>
<td>66</td>
</tr>
<tr>
<td>glutamate</td>
<td>33</td>
<td>66</td>
</tr>
<tr>
<td>glycine</td>
<td>99</td>
<td>198</td>
</tr>
<tr>
<td>taurine</td>
<td>333</td>
<td>666</td>
</tr>
<tr>
<td>GABA</td>
<td>33</td>
<td>66</td>
</tr>
</tbody>
</table>

2.3 Amino Acid measurements

High Performance Liquid Chromatography (HPLC) was used to measure the release of amino acid transmitters from the retina under 'normal' conditions or when depolarised with K⁺ in solutions designed either to allow or prevent Ca²⁺ entry into cells (solutions C-F; see table 2.2). Data was obtained for days 4 to 14 of embryogenesis. The amount of tissue per retina varied with embryonic age and also due to losses during the dissection procedure. These variations were taken into account by expressing the amino acid release as a function of the protein content of the tissue.

2.3.1 Incubation

Retinae of embryos between E4 and E14 were dissected (see chapter 2.A) in bicarbonate-buffered Krebs' solution (Solution C) gassed with 95% O₂/5% CO₂. The whole retina was placed in a small Perspex incubation well and rinsed twice with Krebs' solution. The tissue was incubated in 500 µl of Krebs' solution (or 150 µl at E4) for 30 minutes in a chamber within a rocking water bath held at 32°C. In order to obtain detectable levels of amino acids, several retinae were pooled in each incubation well at earlier stages: at E4
and E6 three retinas, at E8 two retinas, and at E10 to E14 one retina per sample was used. The pH of the Krebs’ solution was kept at 7.36 by flowing gas (95% O₂/5% CO₂) over the incubation wells. Evaporation was minimal (less than 5%) because of the small surface area (200 mm²) of each well. Immediately after incubation the tissue and the incubation solution were separated and stored at -20°C for protein determination and analysis by HPLC, respectively.

### 2.3.2 HPLC analysis

HPLC was carried out with an analytical column (Phase Separation, Spherisorb ODS2 S5) 25 cm long and 4.6 mm in diameter. The column contained silica beads, 5 μm in diameter, which are highly compatible with organic solvents. The column was run in reverse phase mode, which means that the stationary phase (silica beads) is less polar than the mobile phase. The solute (amino acid sample), is retained on the column by the higher hydrophobicity of the stationary phase in comparison to the mobile phase.

The mobile phase generally consists of water or aqueous buffer solutions with water-miscible solvents. The mobile phase, which was used in the experiments described, consisted of two solutions, termed Buffer A and Buffer B, containing water, sodium propionate, methanol and acetonitrile (see table 2.3). All solutions were prepared using distilled de-ionised water and HPLC grade solvents from BDH Chemicals Ltd., Poole, UK. All amino acid standards were obtained from Sigma Chemical Co., St. Louis, MO 63178. The proportion of Buffer A and Buffer B in the mobile phase was not constant throughout one measurement, but changed with time between 0%-100% for each of the solutions. The two solutions were imported with a flow rate of 1 ml/min (maximum pressure 2.07 MPa) and the total running time was 20 minutes. The gradient of the two buffers and their import into the column was controlled by a Jasco pump (PU980). Sample injection was carried out using
an autosampler (Pharmacia 2157). A volume of 25 μl from the amino acid sample was placed into the autosampler and 50 μl of o-phthalaldehyde (OPA) solution were added automatically in order to achieve pre-column derivatisation. This mixture was allowed to react for 1 min and then 50 μl of the mixture was automatically injected into the column. The OPA-reagent was prepared by dissolving 10 mg OPA in 500 μl of methanol and 500 μl of 2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, W1 53233). This was made up to 10 ml with 1 M Na-borate buffer, pH 10.4 (see also table 2.4).

The amino acid solutions, which were used as standard solutions to calibrate the detection sensitivity for each experimental day, contained aspartate, glutamate, glycine, taurine and GABA dissolved in distilled de-ionised water. High concentration stock solutions were stored at -20°C, thawed and diluted with water immediately before HPLC analysis. Two standard amino acid solutions, a "High Standard" and a "Low Standard", were run several times on each experimental day before and during the sample measurements, to give the exact retention times for the amino acids on each day that measurements were made. There was also a small shift in retention times during each day which was monitored and compensated for by running the standard solutions frequently between measurements. Between experimental days the detection sensitivity of the column (fluorescence per pmol) varied slightly; the standard error of all measured values of the standard solutions was 2%-10% of the mean value. Again, this error was compensated for by running the standard solutions several times each day. The "Low Standard" contained 50 pmoles of aspartate, glutamate and GABA; 150 pmoles of glycine and 500 pmoles of taurine in 25 μl. The "High Standard" was made up with exactly twice the amounts of the amino acids. After pre-column derivatisation the autosampler takes 2/3 of the given sample. So the actual amount of amino acid that entered the column was lowered by a third (exact concentrations are given in Table 2.6).
The output from the column was measured with a photomultiplier-based fluorescence detector (FP 920) using an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The photomultiplier transforms the photon flux into a current and the measured values are expressed in mV (see figure 2.2). The amino acid derivatives were identified by their retention times relative to the reference peaks produced by the standard solutions. Concentrations were calculated by comparing peak areas with those of the standard amino acid solutions.

Once the average fluorescence response ($b_{sta}$) of a given amino acid X has been calculated, this value can be used to determine the concentration per area unit of the fluorescence response. This fraction multiplied by the area of the peak gives the amount ($y_{sa}$, [pmoles]) of amino acid X in the injected volume (equation 1). This concentration was corrected for the total incubation volume (500 μl or at E4 150μl) and the amount of protein in [mg] determined for each sample (equation 2) to give the amino acid released by the tissue ($z_{sa}$ [pmoles]).

Equations:

\[
(1) \quad \frac{a_{sta} \text{[pmoles]} \times x_{sa} \text{[area]}}{b_{sta} \text{[area]}} = y_{sa} \text{[pmoles]}
\]

\[
(2) \quad y_{sa} \text{[pmoles]} \times \frac{V_{sa}}{16.67 \mu l} \times \frac{1}{p \text{[mg]}} = z_{sa} \text{[pmoles]} \times ([mg] \text{protein} \times 30[\text{min}])^{-1}
\]

$a_{sta}$: amount of amino acid in the injected standard [pmoles]

$b_{sta}$: fluorescence response of injected standard [peak area]

$x_{sa}$: fluorescence response of injected sample [peak area]
ysa: amount of amino acid in the injected sample [pmoles]
zsa: amount of amino acid in the total sample [pmoles]
Vsa: volume of the total sample (either 500 μl or 150 μl)
16.67 μl: volume of the injected sample

2.3.3 Protein determination

Since the amount of tissue varies with embryonic age and from one dissection to another, the protein content of each sample was determined using a Bio-Rad DC Protein Assay kit.

The theoretical background of the Bio-Rad DC Protein Assay Kit

This is a colourimetric assay for protein concentration following detergent solubilisation. The reaction is similar to the Lowry assay (Lowry et al, 1951) with adaptations that allow 1 hour for measuring the samples in the spectrophotometer with a colour change of less than 5%. In this assay, two reactions lead to the solution becoming coloured; the protein forms a complex with copper in an alkaline medium, and this then forms a coloured reaction product on reduction of the ‘Folin’ reagent. The ‘Folin’ reagent comprises: lithium sulfate, sodium tungstate, sodium molybdate, hydrochloric acid and phosphoric acid. Proteins reduce the ‘Folin’ reagent by loss of 1-3 oxygen atoms, thereby producing several reduced species which have a characteristic blue colour with maximum absorbency at 750 nm.
The gradient program for injection of Buffer A and Buffer B during a run of a single sample was adapted from Turnell and Cooper (1982). The higher proportion of the more polar Buffer A at the beginning of the measurement is responsible for the short retention times for aspartate and glutamate, while the increase in Buffer B from 5 to 9.5 min delays the release of glycine and GABA from the column and at the same time increases the resolution of those peaks.
<table>
<thead>
<tr>
<th>Time [min]</th>
<th>% Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>6.5</td>
<td>70</td>
</tr>
<tr>
<td>9.5</td>
<td>100</td>
</tr>
<tr>
<td>11.5</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2.2

Amino acid chromatogram (lower line) of a "High Standard" solution containing 66 pmoles of aspartate, glutamate and GABA, 198 pmoles of glycine and 666 pmoles taurine. The area of the fluorescence peak relates to the amount of amino acid in the sample. The upper line indicates the proportion of Buffer B in the mobile phase composition.
Peak areas of the ‘High Standard’ solution

The area of a fluorescence peak is directly proportional to the amount of amino acid in the measured sample. Peak areas were calculated using the HPLC machine’s resident Borwin software (area is expressed in arbitrary units), which reports the quantity of amino acid in each peak of the trace. The amino acid derivatives in the samples were identified by their retention times relative to the reference peaks produced by the standard solutions. The concentrations were calculated by comparing peak areas for unknowns with those of the standards (see also equation 1 and 2).
<table>
<thead>
<tr>
<th>Substance</th>
<th>Retention time</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartate</td>
<td>6.61</td>
<td>67,898</td>
</tr>
<tr>
<td>glutamate</td>
<td>8.63</td>
<td>113,659</td>
</tr>
<tr>
<td>glycine</td>
<td>10.38</td>
<td>472,225</td>
</tr>
<tr>
<td>taurine</td>
<td>11.07</td>
<td>1,531,092</td>
</tr>
<tr>
<td>GABA</td>
<td>13.26</td>
<td>170,581</td>
</tr>
<tr>
<td>unknown</td>
<td>14.58</td>
<td>71,547</td>
</tr>
</tbody>
</table>
**The experimental procedure**

Prior to protein assay, the retinal tissue, stored in 200 µl of Krebs' solution (Solution C in Table 2.2), was mechanically disrupted with an Eppendorf homogeniser. To permeabilise the cells, a solution containing detergent (Solution G in Table 2.5) was added to the tissue sample and the cells rinsed up and down in a Pasteur pipette.

A mixture of sodium hydroxide (reagent A of the Bio-Rad DC Protein Assay Kit) and sodium dodecylsulfate (reagent S) 50:1 was freshly prepared (called 'working reagent A') because precipitation occurs within hours after mixing. To 100 µl of 'working reagent A' 20 µl of the protein sample were added and the solution vortexed. Three aliquots were taken from each protein sample, analysed and averaged for the final result. Then each of these aliquots was mixed with 800 µl of Folin reagent (reagent B) and mixed thoroughly. After 15 minutes absorbencies can be read and are stable for 1 hour.

For calibration of the protein assay, a standard curve was produced using bovine serum albumin (BSA, Sigma) at concentrations between 0.1-1.0 mg/ml in distilled water (see Figure 2.4). These standards and a protein-free water sample were processed as described above. Absorption was measured in a spectrophotometer (Pharmacia LKB, Ultrospec 2) at 750nm. The protein-free sample was taken as the zero reference point. From the slope of the standard curve ($a_{sta}$) and the optical density of a sample ($y_{al}$) the total amount of protein per sample can be calculated as follows:

\[
(3) \quad \frac{y_{al}}{a_{sta}} = x_{al \text{[mg]}}.
\]
Figure 2.4

A standard curve for calculation of the protein content

An example of a standard curve made for the protein determination of 7th of March 1997 using 6 different concentrations of BSA (0.1, 0.3, 0.5, 0.7, 0.9, 1.0 mg ml$^{-1}$). Three aliquots of each concentration were taken and measured in the spectrophotometer. The average optical density is plotted as a function of BSA concentration. The line is a least squares fit to the data.
Standard curve

optical density

BSA [mg/ml]

0.00 0.20 0.40 0.60 0.80 1.00
2.4 Patch clamp recordings

The whole-cell patch clamp technique (Hamill et al. 1981) was used to study voltage- and transmitter-gated currents in ganglion cells of the embryonic chick retina. Currents due to the application of an amino acid transmitter reveal the presence of the appropriate receptors in the recorded cell or in cells that have synaptic inputs into it. In this series of experiments, the time of the appearance of glutamate, GABA, and glycine receptors were investigated.

2.4.1 Experimental procedure and materials

Before starting the experiment patch-pipettes were pulled on a BBCH puller (Mecanex, Geneva) from thick-walled borosilicate glass with an internal filament (Clark Electromedical Instruments No. GC150F10), these were sometimes fire-polished to facilitate the formation of a high resistance seal. Thick-wall glass obviates the necessity to Sylgard electrodes, because it reduces the electrode capacitance such that it can be readily compensated for by the patch clamp amplifier capacitance compensation facility. The resistance of electrodes measured in Plain Avian Ringer before sealing onto a cell was usually between 10 and 20 MΩ.

Recordings were made from cells in the GCL of retinas that were acutely dissected or had been stored in oxygenated Ringer (Solution B) not more than 3 hours. The retina was placed flat with the GCL upwards in a Perspex perfusion chamber of about 1.5 ml volume. The retina was held in place by a "harp" made of a platinum wire and very fine nylon strings. In three or four small areas the bundles of axon fibres along with any extracellular matrix were pulled aside to give access to the somata located in the GCL beneath. The perfusion chamber was then mounted on the stage of an upright Zeiss microscope and the cells observed under Hoffman contrast optics. Patch
electrodes were manoeuvred down to the cell to be recorded using a Perspex electrode holder (Clark Electromedical) and a Narishige micromanipulator. A slight positive pressure was applied to remove any external solution that may have accumulated in the tip. The electrode was then pushed up against the soma membrane and gentle suction applied to form a high resistance seal between the tip of the electrode and the cell membrane. At the same time the pipette voltage was lowered slowly from 0 to -60 mV. After 30 to 60 seconds, an additional pulse of suction was applied that ruptured the membrane beneath the pipette tip allowing access to the cell interior. During entry to the whole-cell mode 10 mV pulses were applied and capacity transients were recorded and stored for later analysis. The cell could then be voltage-clamped with the current being measured as the voltage dropped across the 500 MΩ resistance of the current to voltage converter contained within the amplifier headstage (Axopatch 1-C, Axon Instruments, Foster City, CA, USA). The indifferent electrode was an Ag/AgCl pellet contacting the bath solution.

2.4.2 Cell capacitance and series resistance

The cell membrane can be treated as a parallel combination of a capacitor (C_m) and a resistor (R_m). In addition there is a series resistance (R_p) in the pathway from the patch pipette to the cell membrane, which causes a difference between the voltage across the cell membrane and the command voltage (figure 2.5A). In order to measure this voltage error it is necessary to establish the series resistance (R_p). Both the series resistance and the cell membrane capacitance were measured from the current response to a 10 mV voltage step from a holding potential near to the zero current potential. The current transients resulting from this voltage pulse (figure 2.5B) were fitted by a single exponential using a curve fitting computer program (Clampfit, Axon Instruments). The current change can be predicted with the following equation:
\[ l(t) = V(1 + R_m e^{-t/\tau} / R_p)/(R_m + R_p) \]

V: magnitude of the voltage step  
\( R_m \): membrane resistance  
\( R_p \): pipette series resistance  
\( \tau \): time constant of the capacity current decay  
\( t \): 0 at the onset of the voltage step

The Clampfit program measures the initial pipette current \( I_p(0) \), the steady state pipette current at the end of the pulse \( I_p(\infty) \) and the time constant \( \tau \). The ratio between the voltage step and the current at time zero gives the series resistance:

\[ R_p = V / I_p(0). \]

The steady state current (at time=0) is:

\[ I_p(\infty) = V / (R_m + R_p). \]

From these two values the membrane resistance can be calculated:

\[ R_m = (V / I_p(\infty)) - R_p \]
Figure 2.5

Cell membrane properties

A. The circuit equivalent of a whole-cell clamped cell. $R_m$ is the membrane resistance, $R_p$ is the pipette resistance and $C_m$ is the membrane capacitance.

B. The current response of a retinal ganglion cell to a 10 mV depolarisation at a holding potential of -60 mV.
A

B

$\Delta I(0)$

2ms

100pA

$\Delta I(\infty)$
and the cell membrane capacitance can be calculated as follows:

\[
C_m = \frac{\tau (R_m + R_p)}{R_m R_p}.
\]

If the membrane resistance is much larger than the pipette series resistance, this term becomes \(C_m = \tau / R_p\) (Neher and Marty, 1982). This was true for all cells studied.

2.4.3 Correction for junction potential

When the pipette is lowered into the bath, a junction potential develops across the liquid-liquid interface at the pipette tip. This is due to the different ionic compositions of the two solutions. The magnitude of the junction potential depends on the concentration, charge and mobility of the ions in the internal and external solutions. In the solutions used in this thesis, the Na\(^+\) ions in the extracellular and K\(^+\) ions in the intracellular solution largely compensate for each other's contribution to this potential and the anions in the solution largely determine the junction potential.

The potential was measured and corrected for as described by Neher and Marty (1982). The zero current potential was obtained by using the intracellular solution both inside the pipette and outside in the bath. Then the solution in the bath was exchanged for extracellular medium, while the patch-clamp amplifier was in current clamp mode allowing the change in voltage to be monitored. To prevent changes in the reference electrode potential, a 4M NaCl agar bridge was used to connect the reference electrode to the bath solution. The junction potential was 4 mV for pipettes filled with solution A and solution B as the extracellular medium.
2.4.4 Data acquisition and data analysis

Patch clamp recordings were stored on video tape using a pulse code modulator (Sony PCM 701ES). In parallel, a chart recorder (Gould BS 272) produced an analogue trace on paper. The capacity transients and membrane currents were recorded using a computer equipped with a Labmaster laboratory interface (TL-1, Axon Instruments). Currents recorded from retinal neurons were analysed using P-CLAMP software (Axon Instruments). The Excel 5.0 spreadsheet program was used for calculations and plotting graphs. Figures were produced using Power point 4.0 (Windows 95, Microsoft Office).

2.5 Dye-injections

The pattern of gap-junctional coupling between GCL neurons was investigated by whole-cell patch-clamping of cells using pipettes filled with a mixture of fluorescein-dextran (mol. wt. 10KD, 32 mg ml⁻¹) and Neurobiotin (mol. wt. 323D, 5 mg ml⁻¹). The low molecular weight Neurobiotin is free to diffuse through gap junctions into any coupled cells, while the high molecular weight fluorescein-dextran remains trapped within the injected cell. After histochemical treatment to reveal the Neurobiotin the tissue was examined by confocal microscopy.

2.5.1 Dye-injections using whole-cell patch clamping

A piece of retina prepared as described in Section 2.1 was placed GCL uppermost in a Perspex perfusion chamber designed to fit on the microscope stage. The tissue was held down with a ‘harp’ made from a platinum wire and fine nylon threads and prepared as described in section 2.1. Patch pipettes with relatively small tips (pipette resistance (R_pip) ~20 MΩ) were used to
facilitate their removal from the ganglion cell membrane without pulling away the cell body. Pipettes were filled with the two dyes dissolved in intracellular medium (Solution A in Table 2.1). After entering whole-cell mode, two minutes were allowed for the dyes to diffuse into the cell before removing the pipette. Done carefully, the membrane sealed over and the cell body was left intact. After recording up to four cells in each square of retina the tissue was left for 20 minutes for the Neurobiotin to diffuse into any coupled cells.

2.5.2 Histochemistry

The tissue was processed to render the Neurobiotin visible in the confocal microscope using a protocol adapted from Becker and Davies, 1995. Retinae were fixed in 4% paraformaldehyde (TAAB Laboratories Equipment Ltd., Reading, UK) (~12h at 4°C), rinsed three times in phosphate-buffered saline (PBS) (10mins per wash) and then permeabilised in 0.1 M Lysine and 0.1% Triton-X 100 in PBS (1h at room temperature). The tissue was washed free of permeabilisation solution with PBS (3 changes of 10 mins each) and incubated in 1:100 CY3 or CY5 labelled avidin (12h at 4°C). The unbound CY3/5-avidin was removed by washing in PBS (3 changes 10mins each) and the retina mounted in Citifluor (City University) with the GCL uppermost. Coverslips were sealed using nail-varnish.

2.5.3 Image acquisition and analysis

The retinae were examined using a Leica TCS 4D laser scanning confocal microscope. The 494 nm line of the argon-krypton laser was chosen to reveal the recorded cell filled with the fluorescein dextran (emission wavelength: 520 nm) and the Neurobiotin filled coupled cells were visualised using the 550 nm line for CY3 or the 650 nm line for CY5 (emission wavelengths: 565 nm and 670 nm, respectively). Using these wavelengths, separation of the
fluorescence signals from the two dyes was almost complete. A series of 10-130 optical sections (depending on the dendritic tree size and the extent of coupling) about 1 μm apart were taken through the depth of the flat-mounted retina.

The images obtained in the confocal microscope were first displayed as a so-called ‘gallery’ containing all optical sections. These could then be transformed, using the confocal’s resident Scanware 5.0 software, into a two-dimensional ‘projection’, which displays all the information from the thickness of the retina as if seen in a flat-mount. These optical sections could also be turned through 90° to give a ‘rotation’ (a side-view of the retina). ‘Projections’ and ‘rotations’ were then transferred to a Macintosh computer and processed using Adobe’s Photoshop 3.0 and Microsoft’s Power Point 7.0 software. Occasionally it was necessary to omit the most vitread of the optical sections from the stack, because the fluophore (CY3 or CY5) became bound to the AFL obscuring details below.

2.6 Statistical Analysis

All data is presented as the mean ± standard error. In chapter 3, ‘The release of amino acid transmitters in the embryonic retina’, the data from the HPLC experiments aimed at determining the change in the release of amino acid transmitter as development proceeds (sections 3.3.2, 3.3.3, 3.3.5 and 3.3.6 were analysed by ANOVA followed by Bonferroni’s Multiple Comparison Test. The data from sections 3.3.4, 3.3.7 and 3.3.8 of the same chapter describing the Ca²⁺-dependence of transmitter release and the effects of the GABA uptake inhibitor (SKF-89976A) on GABA release were assumed to be of equal variance. But since they involve single comparisons of an experiment with the appropriate control, they were tested using the two-tailed Student’s T-Test. Similarly, in case release in Control solution was compared with release in High K⁺, p-values were obtained by using the two-tailed
Student's T-Test. The data in chapter 5, 'Gap junctional communication between ganglion cells and other neurons during development', concerning the change of the number of cells dye-coupled to ganglion cells (section 5.3.3) was compared using the Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test. All tests were performed using Graphpad Prism 2.0 software.
Chapter 3

The release of amino acid transmitters in the embryonic retina

3.1 Introduction

The amino acids glutamate, GABA and glycine are involved in normal cellular function as well as in neurotransmission. These amino acids are thought to play important roles in the development of the central nervous system (Lipton and Kater, 1989; for review see Ben-Ari et al., 1997). In vivo and in vitro experiments have shown that activation of GABA, glycine and glutamate receptors in developing neurons can influence neuronal survival (Allcorn et al., 1996), growth cone behaviour (for review see Kater and Mills, 1990 and 1991) and neuroblast movement (Komuro and Rakic, 1993). For example, treatment of mouse neuroblastoma cells with GABA increases the length and branching of processes as well as the number of cells (Eins et al., 1983). Using an in vitro microchemotaxis assay, rat embryonic spinal cord neurons were shown to migrate towards picomolar concentrations of nerve growth factor (NGF) and femtomolar GABA concentrations (Behar et al., 1994).

Some effects may involve the interaction between neurotransmitters and neurotrophins (Meyer-Franke et al., 1995; for review see Knipper and Rylett, 1997). In cultures of embryonic hippocampal neurons short-term application of GABA or muscimol increases brain-derived neurotrophic factor mRNA (BDNF mRNA) expression (Berninger et al., 1995) and the GABA-induced expression of BDNF has regulatory effects on the phenotype of hippocampal interneurons (Marty et al., 1996).

Many of the effects of amino acid neurotransmitters in development likely result from membrane depolarisation (GABA has been shown to have a
depolarising action on developing neurons in the CNS; see discussion of
this chapter) leading to Ca\(^{2+}\)-entry via voltage-gated Ca\(^{2+}\)-channels. GABA
and glutamate depolarise ventricular cells in early stages of neocortical
development and decrease DNA synthesis (LoTurco et al., 1995). It has
been suggested that activation of glycine receptors, which are expressed
transiently in early neocortical development also, leads to membrane
depolarisation at early times (Flint et al., 1997).

While studies have focused on the effects that amino acid transmitters
have on developing neurons, it is still unclear when and to what extent the
amino acids are released during development. Little is known of the
mechanisms involved in release or how release is regulated. In the
following experiments HPLC has been used to measure amino acid
release from embryonic chick retinae at different times in development. To
gain some insight into the mechanisms involved, these measurements
were made under conditions that either allowed, facilitated or prevented
Ca\(^{2+}\)-influx into cells.

3.2 Methods

The methods employed in this chapter are described in detail in the
methods section 2.3. Retinae were rapidly dissected from 4 to 14-day-old
chick embryos and rinsed briefly in Krebs' solution (solution C, table 2.2)
before each was transferred to 500 µl of Krebs' or a modification of this
solution (see below) held in the wells of a multi-well plate. The multi-well
plate was placed in a rocking water-bath, gassed with 5% CO\(_2\)/95% O\(_2\) and
warmed to 32°C. When younger retinae were used, up to 3 were placed in
the same well and, at E4, the well volume was reduced to 150 µl. The
tissue was incubated for 30 minutes in one of 4 solutions (see table 2.2):
normal Krebs' solution ('Control solution', solution C), high K\(^+\)-Krebs'
solution ('High K\(^+\)', solution D), high Mg\(^{2+}\)-Krebs' solution ('Control/high
Mg\(^{2+}\), solution E) and high K\(^+\)/high Mg\(^{2+}\)-Krebs' solution ('high K\(^+\)/high Mg\(^{2+}\), solution F) before the supernatant was withdrawn and the amino acid concentrations measured in the HPLC (an example for a trace obtained by HPLC analysis is shown in figure 3.A; see Chapter 2.1 for a more detailed description of the methods). In order to remove peaks present due to any contamination of the water used in the preparation of the samples for HPLC, the traces from water blank samples were subtracted from the experimental samples. The quantities of amino acids present in 500 \(\mu\)l of the water used were small (figures are average of 13 samples±SEM): 0.08±0.01 nmoles of aspartate, 0.06±0.01 nmoles of glutamate, 0.24±0.03 nmoles of glycine and 0.05±0.03 nmoles of GABA. In some incubation samples the quantities of amino acids present were too small to be detected (such results are marked n.d. for 'not detectable' in the figures). The protein content of the retinae was determined using a Bio-Rad DC Protein Assay Kit (a modification of the Lowry assay). Amino acid release is expressed as nmoles mg\(^{-1}\) protein in 30 minutes. All measurements are given as the mean±SEM.

3.3 Results

3.3.1 Change in protein content of the retina with embryonic age

226 protein determinations were carried out; 14 for E3, 20 for E4, 64 for E6, 22 for E8, 42 for E10, 20 for E12 and 44 for E14. The average protein content per retina as shown in figure 3.1, increased from 0.01±0.001 mg (at E3) to 3.04±0.066 mg (at E14). The protein content increases most rapidly around E10 suggesting this to be the period of maximum growth.
Figure 3.A

An example of an amino acid chromatogram obtained using HPLC combined with fluorescence detection.

Upper panel: Sample from E6 retinae in Control/high Mg\textsuperscript{2+}. The fluorescence peaks measured as a change in voltage by the photomultiplier are plotted against time. From these values the Borwin software calculates the peak area in an arbitrary unit (see below).

Lower panel: The peaks for aspartate, glutamate, glycine and GABA from the sample trace above were located by comparison with the retention times from the 'High Standard', which was run 25 minutes earlier (5.79, 8.53, 10.19 and 11.71, respectively. The peak areas calculated by Borwin are listed along the retention times of the amino acid.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Retention time</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartate</td>
<td>5.63</td>
<td>8274</td>
</tr>
<tr>
<td>glutamate</td>
<td>8.41</td>
<td>8500</td>
</tr>
<tr>
<td>glycine</td>
<td>10.08</td>
<td>77935</td>
</tr>
<tr>
<td>GABA</td>
<td>12.73</td>
<td>8701</td>
</tr>
</tbody>
</table>
Figure 3.1

The average protein content per retina plotted as a function of embryonic age (see text for details).

The average protein content per retina was 0.01±0.001 mg at E3 (n=14) and rose to 3.04±0.066 mg at E14, n=42. The polynomial trendline fitted to the data suggests the time of highest growth rate to be around E10. (The inset shows the values for E3 and E4 on an expanded scale.)
Increase in protein content per retina during embryogenesis

protein content (mg)

embryonic day

n=44
n=20
n=64
n=14
n=22
n=42
n=20
n=44

0.02

0.04

0

3

4
3.3.2 Glutamate and aspartate release during retinal development

Glutamate is a major excitatory neurotransmitter in the retina and CNS. Activation of glutamate receptors has profound effects on neuronal survival, dendritic outgrowth, synapse formation and gene expression. Figure 3.2 shows that significant concentrations of glutamate and aspartate are released in Control solution from embryonic retinae from the earliest times tested. E4 retinae released 4.60±0.72 nmoles/mg protein (n=6) of glutamate and 3.11±0.31 nmoles/mg protein (n=6) of aspartate during 30 minutes of incubation. Between E4 and E8 the amount of both glutamate and aspartate decreased steeply (p<0.001) to 0.14±0.08 nmoles/mg protein (n=4) and 0.04±0.06 nmoles/mg protein (n=4), respectively. Further decreases in the release of both transmitters led to them being undetectable at E12 and E14. Thus, at the beginning of the period during which synapses form, the levels of excitatory amino acids that accumulate in the extracellular space are low. Presumably this is a reflection of the presence of an efficient uptake mechanism that pumps any transmitter released back into the cells. In addition, there is a smaller proportion of cells that release glutamate at later times, when GABAergic cells express the enzyme to convert glutamate to GABA (see below).

3.3.3 The depolarisation-evoked release of glutamate and aspartate

In order to measure the amount of amino acid transmitter that is released when cells are depolarised, retinae were incubated in High K⁺ (solution D, table 2.2). The ionic composition of this solution causes cells to depolarise and thus all release mechanisms dependent on depolarisation will be activated.

Figure 3.3 shows that at E4 depolarisation caused no significant change in the release of either glutamate (p=0.22) or aspartate (p=0.29), when
The release of glutamate and aspartate in Control solution.

After 30 minutes of incubation at 32°C the release of glutamate and aspartate was measured using HPLC. The release was normalised to the protein content of the retinae used. N.d.=not detected.

Top panel: The amount of glutamate released was highest early in development at E4 (4.60±0.72 nmoles/mg protein). By E12 the amount of glutamate in the incubation volume of 500 µl had fallen to undetectable levels as it was the case at E14.

Bottom panel: Two days into retinogenesis, at E4, a maximum concentration of 3.11±0.31 nmoles/mg protein aspartate was measured. No aspartate release was detectable at E12 or E14.
Release of glutamate in control condition

Release of aspartate in control condition
Release of glutamate and aspartate in High K$^+$.  

Top panel: High K$^+$-evoked release of glutamate is high early in development ($3.28\pm0.72$ nmoles/mg protein, n=6) and lowest at E14 ($1.03\pm0.23$ nmoles/mg protein, n=10). When compared to the release in Control solution, depolarisation of the tissue enhances the release of glutamate to an increasing extent between E6 and E14. At E4 there was no significant change in glutamate release brought about by High K$^+$.  

Bottom panel: High K$^+$ increased the release of aspartate from the retina at all times tested. At E4 the amount of aspartate released in High K$^+$ was 1.4 times greater than that released in Control solution (but not significantly different), while at E14 release was undetectable in Control solution and in High K$^+$ 0.53±0.08 nmoles/mg protein (n=10).
Release of glutamate in high K^+

Release of aspartate in high K^+
compared to Control solution (see figure 3.2 and figure 3.3). In contrast, at E14 the release of both amino acids was raised from undetectable levels in Control solution to \(1.03 \pm 0.23\) nmoles/mg protein (n=10) in High \(K^+\) for glutamate and \(0.53 \pm 0.08\) nmoles/mg protein (n=10) in High \(K^+\) for aspartate. Thus, depolarisation facilitates the release of glutamate and aspartate by an increasing factor during early development. Surprisingly, the total amount of glutamate released in High \(K^+\) decreased between E4 and E14 (from \(3.28 \pm 0.72\) nmoles/mg protein, n=6; to \(1.03 \pm 0.23\) nmoles/mg protein, n=6) (see figure 3.3, top panel). And the same trend was observed for aspartate (from \(4.60 \pm 1.30\) nmoles/mg protein, n=6; to \(0.53 \pm 0.08\) nmoles/mg protein; n=6). It may be of importance that the levels of excitatory amino acids are lower at the onset of synaptogenesis than early in development, since glutamate has been shown to be excitotoxic in the adult retina (Zeevalk et al., 1995; Villani et al., 1997) and the susceptibility of CNS neurons to glutamate-induced excitotoxicity may alter during development (McDonald and Johnston, 1990; Zeevalk and Nicklas, 1992; for review see Verity, 1994).

3.3.4 The Ca\(^{2+}\)-dependence of glutamate and aspartate release

In order to investigate the Ca\(^{2+}\)-dependence of glutamate and aspartate release at different times, experiments were carried out in which retinae were incubated in the presence of high \([Mg^{2+}]_{ex}\) (20 mM) in Control solution (Control/high \(Mg^{2+}\), solution E, Table 2.2) or in High \(K^+\) (High \(K^+/\)high \(Mg^{2+}\), solution F, Table 2.2). The high \([Mg^{2+}]_{ex}\) blocks Ca\(^{2+}\) channels in the plasma membrane and prevents Ca\(^{2+}\) influx and thus vesicular release (Lipton, 1986; and Neal et al., 1994). Experiments were carried out at E6, E10 and E14. As shown in Figure 3.4 (top panel), at E6 the release of glutamate is reduced in Control/high \(Mg^{2+}\) to 30% of the amount measured in Control solution (from \(1.29 \pm 0.24\) nmoles/mg protein to \(0.39 \pm 0.08\) nmoles/mg protein; n=8 and 4, respectively, p=0.01).
The $\text{Ca}^{2+}$-dependence of glutamate release at E6, E10 and E14.

The $\text{Ca}^{2+}$-dependence of the release was tested using a solution high in $[\text{Mg}^{2+}]_\text{ex}$ (20 mM) and low in $[\text{Ca}^{2+}]_\text{ex}$ (0.2 mM). Glutamate release in Control solution was $\text{Ca}^{2+}$-dependent at E6 (release was reduced in Control/high Mg$^{2+}$ by 70% when compared to Control solution, $p<0.05$). At later times the release of glutamate in Control solution and Control/high Mg$^{2+}$ was too low to measure any effect of high $[\text{Mg}^{2+}]_\text{ex}$. The depolarisation-evoked release of glutamate becomes increasingly $\text{Ca}^{2+}$-dependent with time (47% of the release was $\text{Ca}^{2+}$-dependent at E6 compared to 90% were at E14; both reductions are significant at the 5% level).
Ca\textsuperscript{2+}-dependence of glutamate release

**E6**

![Graph showing Ca\textsuperscript{2+}-dependence of glutamate release for E6.](image)

- Control: n=8
- Kreb's+ high Mg\textsuperscript{2+}: n=4
- High K\textsuperscript{+}: n=5
- High K\textsuperscript{+}+ high Mg\textsuperscript{2+}: n=8

**E10**

![Graph showing Ca\textsuperscript{2+}-dependence of glutamate release for E10.](image)

- Control: n=5
- Kreb's+ high Mg\textsuperscript{2+}: n=6
- High K\textsuperscript{+}: n=11
- High K\textsuperscript{+}+ high Mg\textsuperscript{2+}: n=4

**E14**

![Graph showing Ca\textsuperscript{2+}-dependence of glutamate release for E14.](image)

- Control: n=6
- Kreb's+ high Mg\textsuperscript{2+}: n=3
- High K\textsuperscript{+}: n=10
- High K\textsuperscript{+}+ high Mg\textsuperscript{2+}: n=4

n.d. indicates not determined.
At this age, depolarisation-evoked release of glutamate was dependent on Ca\(^{2+}\)-influx to a lesser extent than the release in Control solution. Incubation in High K\(^+\)/high Mg\(^{2+}\) reduced the amount of glutamate being released to 53% (from 3.12±0.68 nmoles/mg protein in High K\(^+\) to 1.66±0.31 nmoles/mg protein; n=8 and 5, respectively; p=0.03). These results suggest that at least 47% of the glutamate release is dependent on extracellular Ca\(^{2+}\) at this age.

Surprisingly, at E10 (figure 3.4, centre panel) glutamate release in Control/high Mg\(^{2+}\) is reduced only by 8% when compared to the release in Control solution (from 0.06±0.03 nmoles/mg protein to 0.05±0.03 nmoles/mg protein, n=6 and 5, respectively); a difference, which is not statistically significant (p=0.80). Since the amount of glutamate released in both conditions is just above the detection threshold, the Ca\(^{2+}\)-dependence may simply not be resolvable under these experimental conditions. In contrast, the depolarisation-evoked release of glutamate at E10 has become increasingly dependent on Ca\(^{2+}\)-influx: 90% of the High K\(^+\)-stimulated release was blocked in High K\(^+\)/high Mg\(^{2+}\).

Two days after synaptogenesis begins, glutamate release in Control solution was below detection threshold and therefore the Ca\(^{2+}\)-dependence of release could not be addressed (figure 3.4, bottom panel). Similar to the situation at E10, at E14 the depolarisation-evoked release was reduced by 89% in High K\(^+\)/high Mg\(^{2+}\) (from 1.03±0.23 nmoles/mg protein to 0.11±0.02 nmoles/mg protein, n=4 and 10, respectively). These data show that High K\(^+\) stimulates glutamate release which becomes increasingly dependent on extracellular Ca\(^{2+}\) towards synaptogenesis, and likely reflect the development of Ca\(^{2+}\)-dependent vesicular release of this transmitter.

Similar results were obtained for the Ca\(^{2+}\)-dependence of aspartate release during development (figure 3.5). The level of High K\(^+\)-evoked release at E6 and E10 was reduced in High K\(^+\)/high Mg\(^{2+}\) by 66% (p<0.01, n=8).
The Ca²⁺-dependence of aspartate release at E6, E10 and E14.

The release of aspartate under depolarising conditions was more dependent on the influx of extracellular Ca²⁺ at E14 than at earlier times. At this age, the High K⁺/high Mg²⁺-evoked release was 94% lower than the release in High K⁺. This Ca²⁺-dependent component of aspartate release was smaller at E10 and E6 (40% and 70% respectively). Aspartate release in Control solution was partially Ca²⁺-dependent at E6 (76% of the release was reduced in Control solution/high Mg²⁺), while at E10 release in Control/high Mg²⁺ was no different to the release in Control solution (0.03±0.02 nmoles/mg protein in both conditions). At E14, the amount of aspartate being released in Control solution and Control/high Mg²⁺ was below the detection threshold.
Ca$^{2+}$-dependence of aspartate release

**E6**

- Control: n=8
- Control/high Mg$^{2+}$: n=4
- High K$: n=5$
- High K$^+$ + high Mg$^{2+}$: n=8

**E10**

- Control: n=5
- Control/high Mg$^{2+}$: n=6
- High K$: n=11$
- High K$^+$ + high Mg$^{2+}$: n=4

**E14**

- Control: n=6
- Control/high Mg$^{2+}$: n=3
- High K$: n=10$
- High K$^+$ + high Mg$^{2+}$: n=4

Note: n.d. = not determined
and 60%, respectively (p=0.12, n=4), while, at E14 96% of the High K+-evoked release was Ca²⁺-dependent (p<0.1, n=4). As for glutamate, the release of aspartate in Control solution had a Ca²⁺-dependent component at E6 (76%, p<0.05, n=8) when compared to Control/high Mg²⁺ (n=4). This component could not be detected at E10, probably because at this time of development the level of aspartate is low and the effects of blocking the Ca²⁺-influx may not be apparent. By E14, aspartate release in Control solution was below the detection threshold.

3.3.5 GABA and glycine release during retinal development

GABA and glycine are the main inhibitory neurotransmitters of the adult retina and play key roles in lateral inhibition in the IPL and OPL. It is generally acknowledged that, during early development, activation of GABA or glycine receptors has a depolarising effect on immature neurons due to their high [Cl⁻]. Thus, GABA and glycine may act synergistically with the excitatory amino acids glutamate and aspartate on immature neurons.

Figure 3.6 (top panel) shows the release of GABA in Control solution. GABA release was not detectable before E8 (top panel, 0.04±0.06 nmoles/mg protein (n=4)). Unlike the other three amino acids, GABA release and accumulation in the extracellular space in Control solution was highest at the time of synaptogenesis although the increase was not significantly different from the values obtained at E8 (E14, 0.25±0.15 nmoles/mg protein; n=6, p>0.05).

Figure 3.6 (bottom panel) shows the amount of glycine released in Control solution to be high at E4 (10.18±1.84 nmoles/mg protein; n=6) and low at E14 (1.33±0.11 nmoles/mg protein; n=6). Although the release of glycine in Control solution is higher than that of glutamate and aspartate, like
Figure 3.6

The release of GABA and glycine in Control solution.

Top Panel: The release of GABA was not detectable at E4 or E6. From E8 to E14 the amount of GABA released from retinae increased about 6-fold from 0.04±0.06 nmoles/mg protein to 0.25±0.15 nmoles/mg protein, however this increase was not statistically different (p>0.05).

Bottom panel: The amount of glycine measured in Control solution was high at E4 (10.18±1.84 nmoles/mg protein) and decreased between E4 and E6 by 72% and reaching its lowest level at E14 (1.33±0.11 nmoles/mg protein).
Release of GABA in control conditions

Release of glycine in control condition
these transmitters the release of glycine in Control solution decreases with time reaching its lowest level at E14. However, the release of glycine can still be detected at the beginning of synaptogenesis (1.33±0.11 nmoles/mg protein at E14; n=6).

3.3.6 Depolarisation-evoked release of GABA and glycine

In contrast to the excitatory amino acids, K⁺-stimulated release of GABA and glycine (figure 3.7) increased in total and in comparison to the release in Control solution between E4 and E14 (at E14 glycine release was 11-fold higher than in Control solution and GABA release 30-fold, p<0.001 in both cases). The release of GABA in High K⁺ increased 56-fold (p<0.001) between E4 (0.14±0.06 nmoles/mg protein, n=6) and E14 (7.83±1.08 nmoles/mg protein, n=10). The amount of glycine released in High K⁺ at E14 was 4-fold of that at E4 (13.54±2.36 nmoles/mg protein and 3.25±0.46 nmoles/mg protein, n=10 and 6, respectively; p<0.01). Thus depolarisation increases the release of GABA to a greater extent than that of glycine.

3.3.7 The Ca²⁺-dependence of GABA and glycine release

In these experiments the release of GABA and glycine in Control solution and in High K⁺ (solution C and D, Table 2.2) was compared to that in Control/high Mg²⁺ and in High K⁺/high Mg²⁺ (solution E and F, Table 2.2) at E6, E10 and E14. The high extracellular concentration of Mg²⁺ and low concentration of Ca²⁺ is thought to prevent Ca²⁺-influx through voltage-gated Ca²⁺-channels and thus, any transmitter in the medium is likely to be released by Ca²⁺-independent mechanisms. Figure 3.8 compares the release of GABA in Control solution and High K⁺ with the corresponding release in Control/high Mg²⁺ and in High K⁺/Mg²⁺. At E6, the amount of
Figure 3.7

Depolarisation-evoked release of GABA and glycine between E4 and E14.

Top panel: High K⁺-evoked release of GABA increased 56-fold (p<0.001) between E4 (0.14±0.06 nmoles/mg protein) and E14 (7.83±1.08 nmoles/mg protein).

Bottom panel: The amount of glycine released in High K⁺ increased 4-fold (p<0.01) between E4 and E14 (from 3.25±0.46 nmoles/mg protein to 13.54±2.36 nmoles/mg protein).
Release of GABA in high K⁺

Release of glycine in high K⁺
GABA in both Control solution and in Control/high Mg\textsuperscript{2+} was hardly detectable (0±0.03 and 0.01±0.01 nmoles/ mg protein, respectively; n=4 in both cases). As a result it was not possible to determine the effect of blocking Ca\textsuperscript{2+}-influx on transmitter release at this age. At E10, the amount of GABA released in Control solution was low (0.07±0.01 nmoles/mg protein) and showed no Ca\textsuperscript{2+}-dependent component (release in Control/high Mg\textsuperscript{2+} was higher, 0.38±0.27 nmoles/mg protein, than in Control solution, but this increase is not statistically significant (p=0.38). At E14, the release of GABA in control conditions was not significantly different to that released in Control/high Mg\textsuperscript{2+} (p=0.58). Again, the levels of GABA released were very low and any Ca\textsuperscript{2+}-dependent release lost in the noise inherent in the measurements.

In contrast, the depolarisation-evoked release of GABA became increasingly Ca\textsuperscript{2+}-dependent between E6 and E14. While there was no Ca\textsuperscript{2+}-dependent component detectable at E6, by E10 High K+/high Mg\textsuperscript{2+} reduced the amount of GABA being released from the tissue to 35% of that in High K+. At E14 the effect was even greater, when GABA release in High K+/high Mg\textsuperscript{2+} was reduced to 28% of that in High K+ (7.83±1.03 nmoles/mg protein High K+; n=10). The increase in Ca\textsuperscript{2+}-dependent release of GABA at E14 was significant at the 1% level. An increasing Ca\textsuperscript{2+}-dependent component of GABA release during development suggests a growing contribution of vesicular release with time.

Figure 3.9 shows the Ca\textsuperscript{2+}-dependent component of glycine release. At E6 the release of glycine was decreased in Control/Mg\textsuperscript{2+} by 84% compared to amount measured in Control solution (2.82±0.89 nmoles/mg protein). And in High K+/Mg\textsuperscript{2+} the reduction was 57% (from 7.19±1.37 nmoles/mg protein in High K+ to 3.14±0.71 nmoles/mg protein in High K+/Mg\textsuperscript{2+}). This decrease was significant at the 5% level. Surprisingly, at E10 the Ca\textsuperscript{2+}-dependent component of glycine release is apparently lost, since in both,
The Ca\textsuperscript{2+}-dependence of GABA release at E6, E10 and E14.

At E6 the amounts of GABA measured were too small to reveal any effect of the high Mg\textsuperscript{2+}-concentration neither in Control solution nor in High K\textsuperscript{+}. At E10, the High K\textsuperscript{+}-evoked release was reduced by 65% (n=4; p=0.15) in High K\textsuperscript{+}/high Mg\textsuperscript{2+}, while there was no reduction of GABA release in Control/high Mg\textsuperscript{2+} when compared to Control solution. At E14 release of GABA in High K\textsuperscript{+}/high Mg\textsuperscript{2+} was 73%, (n=4; p<0.01) smaller than in High K\textsuperscript{+}. 
Ca$^{2+}$-dependence of GABA release

E6

![Graph showing GABA release at different stages of development](image)

E10

![Graph showing GABA release at different stages of development](image)

E14

![Graph showing GABA release at different stages of development](image)
The Ca$^{2+}$-dependence of glycine release at E6, E10 and E14.

At E6 glycine release was decreased in Control/high Mg$^{2+}$ by 84% (0.46±0.07 nmoles/mg protein, n=4; p<0.1) when compared to Control solution (2.82±0.89 nmoles/mg protein, n=8). In High K*/high Mg$^{2+}$ release of glycine was 44% (from 7.19±1.37 nmoles/mg protein, n=8; p<0.05) of that in High K* (3.12±0.71 nmoles/mg protein, n=5). Similarly, at E14 the High K* -evoked release (13.54±2.36 nmoles/mg protein, n=10) was reduced by 49% in High K*/high Mg$^{2+}$ (6.62±0.34 nmoles/mg protein, n=4; p<0.1). The release of glycine in Control solution showed no Ca$^{2+}$-dependent component either at E14 or at E10, and even the High K* -evoked release was hardly affected by High K*/high Mg$^{2+}$ at E10 (from 10.18±1.75 nmoles/mg protein to 11.54±0.65 nmoles/mg protein, n=11 and 4, respectively; p=0.36).
Ca^{2+}-dependence of glycine release

**E6**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Release in 30 minutes (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>Control/high Mg^{2+}</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>High K^+</td>
<td>5 ± 1.2</td>
</tr>
<tr>
<td>High K^+ + high Mg^{2+}</td>
<td>8 ± 1.5</td>
</tr>
</tbody>
</table>

**E10**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Release in 30 minutes (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>Control/high Mg^{2+}</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>High K^+</td>
<td>6 ± 1.2</td>
</tr>
<tr>
<td>High K^+ + high Mg^{2+}</td>
<td>11 ± 2.1</td>
</tr>
</tbody>
</table>

**E14**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Release in 30 minutes (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 ± 0.8</td>
</tr>
<tr>
<td>Control/high Mg^{2+}</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>High K^+</td>
<td>10 ± 1.5</td>
</tr>
<tr>
<td>High K^+ + high Mg^{2+}</td>
<td>4 ± 0.5</td>
</tr>
</tbody>
</table>
Control/high Mg\textsuperscript{2+} and in High K\textsuperscript{+} high Mg\textsuperscript{2+}, the release of glycine was increased rather than decreased (by 37% and 14% respectively).

At E14, the release of glycine in control conditions was not significantly affected by the Mg\textsuperscript{2+}-block (p=0.06). It is possible that the presence of a highly efficient glycine uptake mechanism could mask the amount of actual release and thus the Ca\textsuperscript{2+}-dependent component. This possibility is supported by the fact that 49% of the depolarisation-evoked release of glycine at E14 is Ca\textsuperscript{2+}-dependent, where the high [K\textsuperscript{+}]\textsubscript{ex} outside would prevent an efficient operation of the uptake carrier, although this reduction is only of marginal statistical significance (p<0.1).

3.3.8 The effect of the GABA uptake blocker SKF89976-A

SKF89976-A is a non-competitive blocker of the neuronal GABA uptake carrier and prevents both the forward and the reverse operation of the carrier. 100 μM SKF76899-A increased the amount of GABA released into the incubation medium (figure 3.10) under normal ionic conditions at all ages, an effect consistent with the forward operation of the GABA transporter. At E6 the release of GABA in Control solution was undetectable in the absence of SKF89976-A (n=4), but rose to (0.43±0.07 nmoles/mg protein, n=3) in its presence. At E10, 0.07±0.01 nmoles/mg protein (n=5) of GABA was released in Control solution and 1.55±0.51 nmoles/mg protein (n=3) in the presence of the uptake blocker. At E14, 0.25±0.03 nmoles/mg protein (n=6) of GABA were released in Control solution and more than twice as much when SKF89976-A was added (0.63±0.03 nmoles/mg protein, n=3) to the Control solution. The increase in GABA release brought about by SKF89976-A was significant at the 1% level at E6 and E10, and at the 0.01% level at E14. These results show that the amount of GABA present in the extracellular medium is reduced by uptake under normal ionic conditions at these times.
Under depolarising conditions (in High K*), SKF89976-A increased the release of GABA by 32% from 0.34±0.13 nmoles/mg protein (n=5) to 0.45±0.04 nmoles/mg protein (n=4) at E6. However this increase was not statistically significant (p=0.49). At E10 there was a reduction in the release of GABA in the presence of SKF89976-A (4.88±1.22 nmoles/mg protein, n=11, and 0.43±0.07 nmoles/mg protein, n=4), however, this was not statistically significant (p=0.48). There was a significant decrease in GABA release in the presence of the uptake blocker at E14, when the release in High K* (7.83±1.08 nmoles/mg protein, n=10) decreased to 4.98±0.44 nmoles/mg protein (n=10; p<0.03) in the presence of SKF89976-A. The reduction in GABA release seen in High K* in the presence of SKF89976-A is consistent with the notion that SKF89976-A blocks the component of GABA release, that is due to reversal of uptake. Reversed operation of the GABA transporter would be expected to occur under the ionic conditions employed in these experiments (for review see Attwell and Mobbs, 1994).

3.4 Discussion

3.4.1 Amino acid neurotransmitters in development

In the developing mammalian brain, it has been shown that neuronal precursors, whose progeny are destined for different cortical layers, have different times of final cell division (Angevine, 1970; Rakic, 1974). The cortical precursor's final exit from the cell cycle is a carefully orchestrated event and is most probably elicited by environmental factors. LoTurco et al. (1995) have shown that GABA and glutamate can elicit depolarising currents in ventricular zone cells of the embryonic rat.
Comparison of GABA release in the presence of the uptake blocker SKF89976-A in either Control solution or High K⁺ at E6, E10 and E14.

Blocking GABA uptake increases the amount of GABA in the extracellular space under control conditions at 6, E10 and E14 (the increase was significant at the 1% level at E6 and E10), while depolarisation of the tissue in High K⁺ is decreased in the presence of SKF89976-A at E10 and E14. This is consistent with the notion that the GABA transporter blocked by SKF89976-A (GAT-1) is present at this time of development and operates in the forward direction under normal ionic conditions, but reverses under depolarising conditions.
Effects of SKF89976-A on GABA release

E6

Release in 30 minutes (nmol/mg protein)

Control | Control/ SKF89976-A | High K⁺ | High K⁺/ SKF89976-A
--- | --- | --- | ---
n=4 | n=3 | n=5 | n=4

n.d.

E10

Release in 30 minutes (nmol/mg protein)

Control | Control/ SKF89976-A | High K⁺ | High K⁺/ SKF89976-A
--- | --- | --- | ---
n=5 | n=3 | n=11 | n=4

E14

Release in 30 minutes (nmol/mg protein)

Control | Control/ SKF89976-A | High K⁺ | High K⁺/ SKF89976-A
--- | --- | --- | ---
n=6 | n=3 | n=10 | n=10
neocortex. Furthermore, the membrane depolarisation evoked by GABA, glutamate or high $K^+$, which is followed by an increase in $[Ca^{2+}]$, through $Ca^{2+}$-influx via voltage-gate channels, which leads to a decrease in DNA synthesis in these cells. LoTurco et al. suggest that amino acid neurotransmitters can influence the neocortical progenitor cell cycle and may thus through an effect on when cells leave the cycle, have profound effects on cell fate.

Later in development, during the migration and differentiation of new-born neurons, excitatory and inhibitory amino acids have a variety of effects. *In vitro* studies have shown that GABA and glutamate can regulate the outgrowth of neurites (Schousboe and Redburn, 1995; Brewer and Cotman, 1989; Lipton and Kater, 1989), influence neuronal survival (Balazs et al., 1988; Mount et al., 1993) and that GABA can serve as a chemoattractant for migrating neurons (Behar et al., 1994; Behar et al., 1995; Behar et al., 1998).

A number of studies have shown neurotransmitter receptors to be present before synapse formation (see chapter 4). The experiments described in this chapter were designed to show whether or not amino acid transmitters are released at early times and under what conditions. To measure the release of amino acid neurotransmitters from neuronal tissue, many studies have employed high performance liquid chromatography (HPLC) on either adult brain slices (Roisin et al., 1991; Panter and Faden, 1992; Goldsmith et al., 1995; Li et al., 1996; Phillips and Cox, 1997) or adult retina (Yazulla et al., 1985; Ayoub and Lam, 1985; Neal and Shah, 1990; Neal et al., 1994; Chiba et al., 1997). Developmental studies on amino acid neurotransmitter appearance and synthesis in the retina have been made using homogenised tissue (Kuriyama et al., 1968; Graham et al., 1970; Pasantes-Morales et al., 1972; Ishikawa et al., 1996) or by measuring release from retinal cultures (Altshuler et al., 1993; Rego et al., 1996; Cubrusly et al., 1998). To date, there has been only one study of
transmitter release in the intact embryonic retina (Lentile et al., 1996), in which the release of glutamate, aspartate and GABA was measured on one embryonic day (E9) to investigate the effects of nitric oxide production on transmitter release in chick retinogenesis.

I have monitored the release of amino acid transmitters from the retina during the first two weeks of chick embryogenesis by measuring the amount of amino acid transmitters released into a small volume of incubation medium under various conditions using HPLC.

### 3.4.2. The release and actions of glutamate during retinal development

In the measurements made here in the embryonic chick retina, the basal level of glutamate in the extracellular medium was high at early times in development. At E4, the first embryonic age that was tested, glutamate release in Control solution was at its highest level. Within the following days, the extracellular concentration of glutamate dropped and fell below the detection threshold at E12 and E14.

Lentile et al. (1996) measured glutamate released from E9 embryonic chick retina. In their study, the amount of glutamate that was measured after 30 minutes of incubation in normal Krebs' solution was 0.15±0.01 nmoles/mg protein, a value similar to the average release of glutamate that was measured at E8 in Control solution in the experiments described here (0.14±0.08 nmoles/mg protein). At E10, the level of extracellular glutamate decreased substantially to 0.06±0.03 nmoles/mg protein in the experiments described here, and was thus lower than that measured by Lentile at E9.

Haberecht and Redburn (1996) used gas chromatography and mass spectroscopy to study the release of glutamate from the postnatal rabbit retina. They show a pattern of glutamate release during postnatal
development that is similar to that observed in embryonic chick. In their experiments, Haberecht and Redburn found that glutamate release into the extracellular space is greater at birth than at any later stages. There was a sharp decline during the following 3 days of development such that by the beginning of synaptogenesis levels of glutamate were low and remained so until adulthood.

High extracellular concentrations of glutamate can be caused by a high constant release of glutamate and a low rate of uptake. Therefore, it is important to know how many and which cells contain high levels of glutamate and how many and which glutamate transporters are present at early times. Early in development, most cells of the developing retina contain glutamate as has been shown by Pow et al. (1994) for the embryonic and neonatal rabbit retina. In their study, glutamate-immunoreactivity was found in cytoblastic cells, photoreceptors, bipolar, ganglion and some amacrine cells. Surprisingly, ganglion cells, which are most intensively labelled in adult retinae, are only weakly labelled at early times, while bipolar cells are strongly labelled throughout development. Pow et al. (1994) found no staining for glutamate in horizontal cells at any stage tested. But in another immunocytochemical study on neonatal rabbit retinae, type A horizontal cells were found to be glutamate-immunoreactive (Redburn et al. 1992). Thus, early in their development both, retinal cells which will become GABAergic (amacrine and horizontal cells) as well as those which will be glutamatergic in the adult (photoreceptors, bipolar and ganglion cells) contain endogenous pools of glutamate. This suggests, that in proportion more cells in the developing than in the adult retina can release glutamate, once they have established a release mechanism.

In order to explain the high levels of glutamate in future GABAergic cells, Redburn et al. (1992) suggested that the endogenous pools of glutamate in GABAergic cells may serve as a precursor for GABA synthesis. Accordingly, Lam et al. (1980) have found that in neonatal rabbit retina
levels of GABA, and the GABA synthesising enzyme glutamic acid decarboxylase (GAD), are low at birth, and then increase steadily until 80% of the adult levels are reached at P9. Similarly, in the embryonic chick retina, GAD-immunoreactivity is not seen before E10, half way through embryogenesis (Hokoc et al., 1990). Thus, early in development, future GABAergic cells may release glutamate upon stimulation until such time as they express GAD, the enzyme that enables them to synthesise GABA. It follows that most retinal neurons contain and may release glutamate early in development.

Another factor that may contribute to high extracellular glutamate concentrations in early development is the absence of a well developed glutamate uptake system. Although little is known about the expression of glutamate transporters in the developing retina, in the developing rat brain, glutamate transporters were found in ventricular zone cells as well as in immature neurons and glia, each transporter subtype having a specific pattern of expression (Furuta et al., 1997; and see discussion of glutamate release mechanism). In addition to the low number of glutamate transporters present in early development, the efficacy of the transporter may be different from that seen in the adult. Since glutamate membrane carriers open a Cl⁻-channel when operating, and the intracellular Cl⁻-concentration is high in undifferentiated neurons (Fatima-Shad and Barry, 1993), Cl⁻-ions will diffuse out of the cell causing the membrane potential to rise and depolarise the cell. Depolarisation and a change in the ionic gradients of Na⁺ and K⁺-ions will tend to reverse the cycle of the carrier resulting in a release of glutamate from the cell (Attwell et al., 1993; Attwell and Mobbs, 1994; for review see Takahashi et al., 1997). The reversed cycle of the glutamate transporters is favoured by the high intracellular concentration of glutamate in GABAergic neurons until GAD is expressed (see above) and in undifferentiated Müller cells until glutamine synthetase (GS) is present to transaminate glutamate into glutamine. In the developing retina, GS is not expressed until late in development (in chick
retina from E15 onwards [Prada et al., 1998] and in rabbit retina from P8 onwards [Fletcher and Kallionatis, 1997]). Thus, for uptake of extracellular glutamate at early times in development glutamate transporter may have to run against an increased glutamate concentration in the cytoplasm of neuron and glia due to the absence of GAD and GS. The late expression of GS and GAD in the retina could be a major factor contributing to high extracellular concentrations of glutamate in early retinal development.

3.4.3 Mechanisms of glutamate release

Except for the earliest day tested, E4, depolarisation in High K* enhanced the release of glutamate when compared to control conditions. Although the total amount of glutamate released in High K* and in Control solution decreased towards synaptogenesis, the effect of depolarisation on glutamate release became much greater with time and was highest at E12. This increase in the release of glutamate in High K* with time may be due to an increase in the expression of the proteins that are necessary for exocytosis.

Haberecht and Redburn (1996), who investigated the total glutamate content of postnatal rabbit retinas at P1, P3, P5 and adult using gas chromatography and mass spectroscopy, found an increase in the endogenous glutamate pool in retinal tissue during this period. The highest increase in glutamate content occurs between P3 and P5 which coincides with the peak period of synaptogenesis in the rabbit. Similar results were obtained in the developing rat retina (Macaione et al., 1974), where the total glutamate content of the tissue increases sharply between P10 and P15 and from then on moderately until adulthood. Thus, during development the level of extracellular glutamate is not directly linked to the level of glutamate inside cells.
Unlike the results presented here, the study by Haberecht and Redburn on neonatal rabbit retinae (1996) found a depolarisation-evoked increase in glutamate release only at P1 (ca. 2-fold), but no effect at any stage later in development (at P3, P5 and in adult retinae). Since in this study high $[K^+]_{ex}$ solution was applied only as one dose (20 µl of 500 mM KCl added into the bath superfusion system) and the samples of supernatant were taken every 2 minutes, it is possible that the depolarisation-evoked release of glutamate was immediately taken up by high-affinity glutamate transporters before the first sample was taken. Assuming that more glutamate uptake carriers are expressed with time and that these become increasingly efficient, the effect of a brief application of high $[K^+]_{ex}$ will be less effective later in development than at early times.

In a series of experiments of Haberecht and Redburn’s study (1996), the tissue was exposed to D-aspartate, a competitive glutamate uptake blocker, to measure the amount of glutamate released in the absence of glutamate uptake. Since the proportion of glutamate released in the presence of D-aspartate compared to control conditions increased with time, the authors concluded that the overall uptake activity is low at birth, which results in a higher extracellular glutamate concentration in the retina early in development.

Another series of experiments described in this thesis investigated the Ca$^{2+}$-dependence of glutamate release at E6, E10 and E14 to determine the contribution of vesicular release to the pool of glutamate in the extracellular space. While the release in Control solution shows a Ca$^{2+}$-dependent component at E6 (about 70%, see figure 3.5), this component is not detectable at E10 or E14. It is possible that at these later stages of development, glutamate transporters take up glutamate with such efficiency that the difference between glutamate release in Control solution and in Control/high Mg$^{2+}$ cannot be resolved. Incubation in High K$^+$ leads to ionic conditions (high $[K^+]_{ex}$ and high $[Na^+]_{in}$) that favour the reversal of
the transporter cycle causing release of glutamate (for review see Takahashi et al., 1997). Since this release is Ca\textsuperscript{2+}-independent, one would expect an increase in Ca\textsuperscript{2+}-independent release parallel to an increase in the number of transporters. In the experiments described here, the Ca\textsuperscript{2+}-independent component of glutamate release in High K\textsuperscript{+} decreases drastically between E6 and E10 from 53\% to 11\%. Although these results may not give any information on the number of transporters in the tissue, they clearly show an increasing component of vesicular release towards synaptogenesis.

The presence of glutamate transporters in the retina has been studied extensively using immunocytochemistry. In the adult chick retina, as well as in rat, turtle, salamander and goldfish retina, the excitatory amino acid carrier 1 (EAAC1) is present in the IPL and in the somata of bipolar, amacrine and ganglion cells (Schultz and Stell, 1996). EAAC1-immunoreactivity was also found in the rat optic nerve, in addition to immunoreactivity for GLT-1 and GLAST (Choi and Chiu, 1997). The authors suggest that the expression of both glial and neuronal glutamate uptake carrier in the optic nerve indicates an interaction between axons and glia, which is mediated by non-vesicular release of glutamate.

While there is not much information available on glutamate transporters in the developing retina, many studies have focused on their presence in the brain. In the developing rat brain, EAAC1 is the only glutamate transporter that is expressed at a higher level in the new-born brain compared to the adult brain (Furuta et al., 1997). Furuta et al. have shown that the expression of the other carriers, EAAT-4, GLT-1 and GLAST, progressively increases during postnatal development. On the other hand, GLT-1 and GLAST are present at low levels in the foetal rat brain and their mRNA has been detected in ventricular zone cells of the embryonic mouse brain (Shibata et al., 1996). Thus, the expression of glutamate transporter subtypes is differentially regulated during development, suggesting their roles in development may be different from those in the adult.
3.4.4 The release and actions of GABA during retinal development

What are the effects of GABA on developing retinal neurons? *In vitro* experiments with embryonic chick brain and retina have shown that GABA treatment (10 μM) promoted proliferation and differentiation by affecting the length and branching of neurites (Spoerri, 1988). In developing rabbit retina, it has been shown that the presence of GABAergic horizontal cells is necessary for cone development. After lesioning of horizontal cells with kainate, cone photoreceptor terminals disappeared from the OPL, but some cone-like structures were found in the INL, suggesting that the signal for their normal position was missing causing them to differentiate in this inappropriate location (Messersmith and Redburn, 1993). The rod photoreceptors in these experiments maintained their normal position, but produced an abundance of terminals.

Localisation of GABA immunoreactivity (GABA-IR) has been examined in retinae of a variety of animals. In the adult chick retina, amacrine cells and horizontal cells have been shown to be immunoreactive (Agardh et al., 1986) as well as some ganglion cells (Hamassaki-Britto et al., 1991). In the developing chick retina, GABA-IR was observed first at E6 (Hokoc et al., 1990) and was clearly associated with horizontal cells (Araki and Kimura, 1991), amacrine and bipolar cells (Reiss et al., 1997). In chick retinal cultures, approximately 32% of the cells develop into GABAergic cells, putative *in vitro* counterparts of GABAergic amacrine cells (Huba and Hoffman, 1990; Yamashita et al., 1994). Release of GABA in cultures of embryonic chick retinae was detected by de Pomerai et al. (1983).

The HPLC experiments in this chapter have shown that early in development, the level of extracellular GABA is very low. In fact, GABA could not be detected in Control solution before E8. The amount of GABA measured increased steeply at E14. This late increase is not unexpected since GAD-IR is not found in the chick retina before E10 (Hokoc et al.,
Although GABA was detected in the experiments of this thesis a few days before E10, the amounts measured were very low indicating either little expression of GAD, presumably at a level undetectable by antibody-staining, or the existence of a different pathway for GABA synthesis (see below). Thus, during the first half of embryonic development, endogenous GABA levels are extremely low, which is somewhat at odds with the roles for GABA during early development, at times when GABA receptor activation depolarises cells (see discussion of chapter 4).

The HPLC measurements of GABA release from E9 chick retina made by Lentile et al. (1996) yielded values as high as (0.09±0.005 nmol/mg protein in Krebs’ solution after 30 minutes of incubation). Their values are close to those found here at E10 (0.07 ±0.01 nmoles/mg protein, n=5). In their study, the tissue had been preincubated in Krebs’ solution for 60 minutes, which could result in slightly higher values. The prolonged total incubation time may have caused some cells to die. Quantification of the GABA being present in the entire developing rat retina, which has been carried out by Macaione et al. (1974) show that retinal GABA content is very low at birth, but increased steeply between P5 and P15, before and during the early stages of synaptogenesis. These results parallel those for GABA release during chick retinogenesis. However, in Macaione et al’s experiments the total tissue content of GABA was shown to be similar to that of aspartate and higher than that of glycine. In the release experiments described in this chapter, the release of GABA into the extracellular space in embryonic chick retinae was substantially lower than the release of aspartate or glycine (glycine release was 92 times higher than GABA release at E8 and 5 times higher at E14; see also release of glycine in 3.3.5). The high levels of GABA in whole rat retina appear to contradict the late expression of GAD demonstrated by Hokoc et al. (1990).

Immunocytochemistry, while not quantitative, allows identification of GABAergic cells and localisation of GABA within cellular compartments.
Immunostaining of horizontal cells in the developing chick retina reveals immunoreactive processes in the OPL at E9 before the appearance of immunoreactive somata (Araki and Kimura, 1991). In the pigeon retina, mouse monoclonal antibodies to glutaraldehyde-linked GABA labelled densely packed processes in the IPL at hatching with the cell bodies of amacrine and horizontal cells becoming labelled a few days later (Bagnoli et al., 1989). This indicates the presence of GABA in the retina at times when the cells are extending processes. The possibility of GABA being released from extending processes has been proven to be true in a series of studies measuring GABA release from growth cones, which were isolated from neonatal rat forebrain (Gordon-Weeks et al., 1984; Lockerbie and Gordon-Weeks, 1986; Taylor and Gordon-Weeks, 1989). In addition, an immunocytochemical study of the embryonic chick retina (Hokoc et al, 1990), using antisera to both GABA and GAD showed that GABA-positive staining could be detected at E6, but GAD-positive staining could not be detected until E10. This temporal disparity led the authors to suggest that early in development, GABA may be synthesised via an alternative pathway, in which putrescine is a precursor. Putrescine has been shown to be present at high levels early in chick retinal development (Taibi et al. 1994). Similar results have been obtained in new-born rabbit retina, where autoradiographic and immunocytochemical experiments with $^{3}$H-GABA show low but detectable extracellular levels of GABA before the synthesising enzyme GAD is (Lam et al., 1980). During the following period of postnatal development, the increase in GAD expression paralleled that of the GABA-degrading enzyme, GABA-glutamate transaminase and GABA itself. Taking the results of these studies together, it appears that GABA is synthesised in small amounts from putrescine and released from growth cones early in development.
3.4.5 The mechanisms of GABA release during development

When embryonic chick retinae were incubated under depolarising conditions (in High K*), there was a small amount of GABA released into the incubation medium even at E4 and E6, when no GABA release was detected in Control solution. Thus, at E4, a mechanism for GABA release is present and can be initiated by membrane depolarisation. The K*-evoked release of GABA increased steeply between E8 and E10 and remained high until E14, the last day considered in this study. The steep increase at E10, a time two days before synapses start to form in the IPL (Hughes and LaVelle, 1974; Hering and Kröger, 1996) could be due to vesicular release that is not yet localised at synapses. At E7, the two synaptic proteins, synaptophysin and synaptotagmin, are found in the IPL of the embryonic chick retina, in addition to the transient expression of synaptotagmin in the synapse-free AFL at this time suggesting that these proteins are not confined to synapses during early development of the retina (Hering and Kröger, 1996). The experiments, in which Ca2+-entry was blocked by a high [Mg2+]ex in the incubation medium show the development of a Ca2+-dependent component of K*-evoked release, which increases with time. While there was no difference between GABA release in High K* and in High K*/high Mg2* at E6, High K*/high Mg2* reduced release at E10 by 65% and at E14 by 72% compared to High K* (see figure 3.9). Because Ca2+-entry is usually necessary for exocytosis of transmitter-containing vesicles, the results in High K* compared to those in High K*/high Mg2* indicate that vesicular release increases towards synaptogenesis. While GABA release in Control/high Mg2* was not detectable at E6 and therefore the Ca2+-dependent component could not be resolved, the release at E10 and E14 in Control solution was not significantly reduced in the presence of high extracellular Mg2+. However, it is possible that GABA, which had been released during the time of incubation, was taken up almost completely by GABA transporters, so that
the difference between Control solution with and without the high \([\text{Mg}^{2+}]_\text{ex}\) was not noticeable.

In addition to the vesicular release, there was still a substantial amount of Ca\(^{2+}\)-independent release of GABA in the presence of high \([\text{K}^+]_\text{ex}\) at all times tested. Ca\(^{2+}\)-independent release has been reported from isolated growth cones prepared from neonatal rat forebrain (Lockerbie and Gordon-Weeks, 1986). In their study, the Ca\(^{2+}\)-dependent component of GABA release increased with further maturation of the tissue, a result similar to that found here. The Ca\(^{2+}\)-independent component of GABA release in the retina may be explained by a reversal of the uptake carrier. Reversed uptake is favoured by membrane depolarisation and high \([\text{K}^+]_\text{ex}\) (Attwell et al., 1993; for review see Attwell and Mobbs, 1994; Takahashi et al., 1997). These conditions are met in the High K\(^+\) solutions used here.

To investigate the contribution to GABA release by reverse operation of GABA transporters to the GABA release seen in embryonic chick retinae, SKF89976-A was added to Control and High K\(^+\) solution to block the action of the GABA uptake carrier GAT-1 (see figure 3.10). In control conditions, blockade of the GABA uptake carrier increased the amount of GABA which remained in the incubation medium on all three days tested, when compared to Control solution. This result suggests that the GABA released in Control solution in the absence of SKF89976-A is taken up again by GABA transporters. Thus, the amount of GABA measured in Control solution was significantly less than the actual amount of GABA released. In contrast, the uptake blocker in High K\(^+\) reduced the release of GABA from retinae at E10 and E14. Under these conditions, the GABA transporter likely works in reverse mode and blocking its operation would be expected to reduce the amounts of GABA released into the incubation medium. At E6, the quantity of GABA released in high K\(^+\) Krebs' solution was not apparently affected by SKF89976-A. However, the quantities of GABA
released at this time were small and the effects of the uptake blocker may be masked by the noise inherent in the measurements.

It has been suggested by Hollyfield et al. (1979) that in the developing retina of *Xenopus laevis*, neuronal GABA uptake carriers are expressed before GABA synthesis is established. In light-adapted fish and chicken retinae, uptake of gamma-vinyl GABA into H1 horizontal cells has been shown by immunocytochemistry (Pow et al., 1996) and staining was greatly reduced in dark-adapted retinae, suggesting that transport of GABA is dependent on the membrane potential in these cells. In the adult rat retina, GABA plasma membrane transporters (GATs) have been studied using polyclonal antibodies (Johnson et al., 1996). GAT-1 was strongly expressed by amacrine, displaced amacrine and interplexiform cells and weakly by Müller cells (see also Brecha and Weigman, 1994). GAT-3 labelling was found mainly in Müller cells; GAT-2 immunostaining was observed only in the pigment epithelium.

3.4.6 The release and action of glycine during retinal development

In the adult vertebrate retina, glycine, like GABA, is a major inhibitory amino acid neurotransmitter. In the embryonic chick retina, release of glycine under control conditions was high at E4 and decreased towards the time of synaptogenesis. These results are surprising in that this pattern of release is different to that of GABA, but rather similar to the release pattern of glutamate and aspartate, except that the levels of glycine involved are higher. This may be due to the fact that GABA is the only neurotransmitter amongst the four that is not needed in the cell's metabolism and its synthesising enzyme GAD is not expressed until half way through embryogenesis.
Using immunocytochemistry 60% of the amacrine cells and a smaller proportion of bipolar cells have been shown to be glycine-immunoreactive in the adult rabbit retina (Crook and Pow, 1997). Similarly, in the adult goldfish retina a large number of the amacrine and interplexiform cells, that form dendro-somatic contacts onto GABAergic amacrine cells, are glycine-immunoreactive (Yazulla et al., 1996).

In postnatal rabbit retinas, some putative ganglion cells are transiently glycine-immunoreactive (Pow et al., 1994). In the developing rat retina, it has been shown that glycine is present at birth and localised in ventricular zone cells which appear to migrate through the neuroblastic layer (Fletcher and Kallioniatis, 1997). The identity of these cells is unknown.

The generally high background level of glycine may be important for potentiation of NMDA receptor activation, which is thought to play an important role in early development by inducing a longer lasting depolarisation and a greater Ca$^{2+}$-influx than AMPA receptor activation (for review see Ben-Ari et al., 1997). The electrophysiological experiments described in chapter 4 show that ganglion cells express functional NMDA receptors from E8 onwards.

Since glycine has a depolarising effect on developing neurons due to their high [Cl$^{-}$] (Owens et al., 1996), extracellular glycine at early times may result in a tonic depolarisation of cells that express glycine receptors. Hering and Kröger (1996) have investigated the appearance of gephyrin, the protein that anchors glycine receptors at synapses, in the developing IPL of the chick retina and found that it is absent prior to E12. The experiments described in chapter 4 show functional glycine receptors to be present on ganglion cells at much earlier times (E6 onwards) suggesting glycine receptors may be distributed over the entire cell membrane rather than being aggregated at synapses in the IPL at these times. Accordingly, Colin et al. (1996) have shown that the gephyrin is expressed prior to
synapse formation and is present in the cytoplasm and in the somatic plasma membrane of postnatal rat spinal cord neurons. It is not until several days later (P7) that gephyrin accumulates at postsynaptic membranes and disappears from the soma. The ubiquitous distribution of the glycine receptor on ganglion cells indicates a developmental role for glycine receptor activation that is clearly different from synaptic transmission.

3.4.7 The mechanisms of glycine release during development

K+-evoked release of glycine increases towards synaptogenesis, as it does for GABA, while the K+-evoked release of glutamate and aspartate declines. By the time of synapse formation GABA and glycine shunt or hyperpolarise the cell membrane, thus it is not surprising to find that the two inhibitory amino acids show a similar pattern of release at this time which opposes the pattern of the excitatory amino acids, glutamate and aspartate.

The Ca2+-dependent and probably vesicular component of glycine release was revealed in the presence of high [Mg2+]ex. At E6 both the release in Control solution and in High K+ were partially dependent on the extracellular Ca2+. Surprisingly, at E10 the release in neither of the two conditions was affected by high [Mg2+]ex, presumably reflecting a lack of Ca2+-dependent glycine release at this time.

The Ca2+-independent component of glycine release may be due to reversal of glycine transporters (GLYT's). To date, two types of mammalian glycine transporters have been cloned: GLYT1, which is found mainly in glia (Zafra et al., 1995) and GLYT2, which is found in presynaptic membranes of glycinergic neurons (van den Pol and Gorcs, 1988; Wenthold et al., 1988; Jursky and Nelson, 1995). One notable exception to
the almost exclusive expression of GLYT1 by glia is seen in the retina, where it is found in amacrine cells (Zafra et al., 1997).

3.4.8 Further experiments:

Further experiments to investigate the sources of GABA release would involve determining the role of the glial GABA transporter (GAT-3). Unfortunately, the only blocker available is β-alanine, which has a number of other actions, for example activation of the GABA$_C$ receptor (Calvo and Miledi, 1995). β-alanine is a competitive blocker of GABA transport and will not prevent reversed uptake. Since extracellular concentrations of glutamate are high at early times, it would be interesting to know the contribution of reversed uptake to the release of glutamate before vesicular release is established. Unfortunately, the available blockers of glutamate transporters compete with glutamate and aspartate for their binding sites on the transporter and would not prevent reversed uptake.
Chapter 4

The appearance of amino acid transmitter receptors in the embryonic retina

4.1 Introduction

Our knowledge of the temporal sequence in which neurotransmitter receptors and their associated ion channels develop in the vertebrate retina is based mainly on immunocyto-chemical and Ca\(^{2+}\)-imaging techniques (Yamashita and Fukuda, 1993; Yamashita et al., 1994; Sakaki et al., 1996). However, immunocytochemistry cannot distinguish whether or not the receptors labelled are functional and Ca\(^{2+}\)-imaging, which detects transmitter receptors by virtue of the Ca\(^{2+}\)-influx through the receptor itself or its release from intracellular stores, cannot resolve cells in which the receptors shunt or hyperpolarise the membrane. Since most of the imaging techniques applied to the developing retina have not employed confocal microscopy the results obtained have shed little light on the localisation of the receptors detected. In theory, patch-clamping, which can detect single ion channels, should be more sensitive than other techniques and could be used to detect which cells express the receptors.

In the following experiments the whole-cell patch-clamp technique has been employed to investigate the appearance of amino acid transmitter receptors on ganglion cells during chick retinogenesis. Ganglion cells are the first retinal cells to be born and differentiate and are thus likely to express transmitter receptors before other retinal neurons.
4.2 Methods

Electrical recordings were made from retinal ganglion cells between E6 and E16 (see section 2.2 for details). Retinae were dissected from the eye and mounted ganglion cell surface uppermost in a recording chamber. The retina was held flat with a platinum and nylon 'harp' and kept continuously superfused with oxygenated Ringer's solution (solution B, table 2.2) via a gravity-fed perfusion system. A series of reservoirs and taps enabled the Ringer's solution to be changed for one containing drugs. Solution exchanges were typically complete within a few seconds. To enable access to the cell bodies of ganglion cell layer neurons the AFL and any extracellular matrix were removed carefully from several small areas with a pair of fine forceps. The patch-pipette could be manoeuvred through the small gaps created and pushed into contact with ganglion cells with intact axons. The possession of an axon and the larger size of the cell bodies of ganglion cells was used to distinguish them from the smaller amacrine cells present in the ganglion cell layer at later times. The solution in the patch-pipette (solution A, table 2.1) contained a high concentration of Cs⁺-gluconate to block K⁺-channels and thus decrease the noise present in the recordings.

4.3 Results

4.3.1 The passive membrane properties of ganglion cells during retinogenesis

In the experiments to determine the time of appearance of transmitter receptors 210 ganglion cells were recorded. A further 308 cells recorded in dye injection experiments (see Chapter 5) were included in the measurements of cell capacitance. Membrane capacitance was determined from an analysis of the current transients that result from a 10 mV step in the holding potential (see section 2.2). Because the decay of the transient was
well fit by a single exponential, it was assumed that the dendrites did not contribute significantly to the cell's internal resistance.

The mean membrane capacitance, resistance and series resistance were 12.18±0.33 pF, 1257±65 and 39±1 MΩ (n=516), respectively (data from all embryonic ages included). Membrane capacitance changed with embryonic age (see below). Membrane resistance is without physiological meaning under the recording conditions employed since a large fraction of the membrane conductance was blocked by Cs⁺-ions. This measurement is not considered further. Cells employed in the analysis of transmitter-evoked membrane currents showed series resistance voltage errors of less than 5mV.

4.3.2 Changes in membrane capacitance with embryonic age

The change in membrane capacitance as a function of embryonic age is shown in Figure 4.1. The capacitance increased between E6, a time when dye injections show that ganglion cells are largely devoid of dendritic processes, and E12, when synapse formation begins and the cells have developed a complex morphology (the mean capacitance at E6 was 5.5±0.5 pF, n= 25, and at E12, 17.5±1.9 pF, n=22). Membrane capacitance appears to reach a plateau at E12 and does not increase significantly during E14-16.

The range of membrane capacitance at early times was significantly smaller than that seen at later times. For example the mean capacitance at E7 was 7.3±0.5 pF (n=97) with 98% of cells with a capacitance between 2 and 16pF and at E11 it was 15.1±0.6 pF (n=176) with 98% of cells with a capacitance between 2 and 32pF (figures 4.2A and 4.2B). This finding is consistent with the increase in membrane capacitance occurring through the elaboration of dendritic processes during this period (see chapter 5 for details). The ganglion cell population consists of a limited number of different
Figure 4.1

The change of ganglion cell capacitance between E6 and E16.

The mean capacitance (standard errors are shown) increases between E6 (5.5±0.5pF) and E12 (17.5±1.9pF) at which time it appears to reach a plateau.
Change in ganglion cell capacitance with embryonic age

$C_m \ (pF)$
Figure 4.2

A) The distribution of ganglion cell membrane capacitance at E7.

There is a near unimodal distribution of membrane capacitance. 77 out of 86 cells had a membrane capacitance between 2 and 16pF and only 2 cells had a membrane capacitance outside this range. Mean capacitance was 7.3±0.5 pF.

B) The distribution of ganglion cell membrane capacitance at E11.

The mean capacitance at E11 (15.1±0.6 pF, n=176) is higher than that at E7 (7.3±0.5 pF) and the range is much wider (98% of cells with a capacitance between 2 and 32pF.)
Ganglion cell capacitance at E7

Ganglion cell capacitance at E11
morphological classes (see Chapter 5) with different dendritic areas and cell body diameters. Consistent with this, the histogram of cell capacitance at E11 is broader than at E7. However, distinct peaks are absent possibly because of the presence of different ganglion cell classes, variations in, and overlap between the membrane area within these.

4.4 Glutamate-evoked currents in embryonic ganglion cells

The excitatory amino acid transmitter glutamate plays an important role in the development of the CNS including the retina (for review see Kaczmarek et al., 1997 and McDonald and Johnston, 1990). NMDA receptors, which are Ca$^{2+}$-permeable Na$^+$/K$^+$ channels can exert a direct effect on the levels of cytoplasmic Ca$^{2+}$ (MacDermott et al., 1986; Ascher and Nowak, 1988). Electrophysiological studies of the embryonic and postnatal retina of the rat have demonstrated the presence of AMPA/kainate receptors several days before synaptogenesis begins (Rörig and Grantyn, 1993). Biochemical studies have shown that glutamate receptors are present in the embryonic chick retina after E7 (Somahano et al., 1988) and in Ca$^{2+}$-imaging experiments from E7 onwards activation of these receptors causes an increase in [Ca$^{2+}$]$_i$ (S. Allcorn PhD thesis, 1995).

In the following series of patch-clamp experiments on E6 to E14 retinae the synthetic agonists NMDA (50 μM, Tocris) and AMPA (100 μM, Tocris) were used to distinguish between NMDA and non-NMDA receptors. The appearance of glutamate receptors was investigated by bath application of the agonist and recording of the membrane current under voltage-clamp. To release the Mg$^{2+}$-block of the NMDA receptor cells were depolarised to ~-45 mV. The contribution of other receptor types to the current response was tested by application of the specific blockers, AP5 (50 μM, Tocris) and CNQX (50 μM, Tocris) respectively.
4.4.1 The developmental profile of NMDA receptor-mediated currents

Current responses to the application of NMDA were absent from ganglion cells in E6 retinae (n=11). This is perhaps a surprising result given the importance of NMDA channels in neuronal development shown for example for the migration of granule cells in cerebellar development (Komuro and Rakic, 1993; Rossi and Slater, 1993; for review see Rakic and Komuro, 1995). However, by E8 84% (n=19) (see figure 4.3) of the cells tested responded to application of NMDA with an inward current at holding potentials of ~-45 mV. There was no systematic increase in the fraction of cells responding between E10 and E14.

At E12 application of NMDA produced currents in all of the ganglion cells that were recorded (n=12). The currents were inward at negative holding potentials ($V_h$) and outward at positive holding potentials (see figure 4.4A). The reversal potential for the NMDA-evoked current was $-3.5\pm3.5$ mV (n=5). The NMDA-evoked current in ganglion cells at E12 was reversibly blocked by co-application of AP5 (current reduced by 95±3.2% (n=4)). Figure 4.4B shows an example of this block at a holding potential of -45 mV.

4.4.2 The developmental profile of non-NMDA receptor mediated responses

The presence of non-NMDA receptors was tested through application of AMPA, which acts as an agonist at non-NMDA receptors. Bath application of 100 µM AMPA produced current responses in 18% of cells tested at E6 (n=11) (figure 4.5). At E8 83% (n=23) of cells showed AMPA-evoked currents. These data show that the majority of ganglion cells start to express AMPA receptors within a small temporal window between E6 and E8. Similarly high percentages of cells responded at E10, 12 and 14.
The NMDA responses first appear at E8. Cells were patch-clamped in whole-cell mode and held at relatively positive potentials (-45 mV) to release any Mg\textsuperscript{2+}-block of the receptor. Bath application of NMDA did not produce a current in any of the cells responded at E6 (n=11). However, between E8 and E14 the majority of cells responded.
Ganglion cells responding to NMDA during embryogenesis

% of cells responding

E6  E8  E10  E12  E14

n=11  n=19  n=8  n=12  n=13
Figure 4.4

A) Voltage-dependence of the current evoked by 50 μM NMDA in a retinal ganglion cell at E12.

Left-hand panel: The peak NMDA current plotted as a function of membrane potential. The reversal potential of the current in this cell was -3.7 mV, the average reversal potential was -3.5±3.5 mV (n=5).

Right-hand panel: The membrane currents evoked by 50 μM NMDA at a series of different voltages (taken from data shown in left-hand panel). The holding potential is indicated next to each trace and the timing of NMDA application is shown by bars.

B) Blockade of the NMDA-evoked current by AP5.

Left-hand panel - the NMDA-evoked current in a ganglion cell of an E11 retina. Centre panel - complete blockade of the current in response to 50μM NMDA by co-application of 50 μM AP5. Right-hand panel - the current response to NMDA after a 2 minute wash in Ringer's solution.
A

B

137
Figure 4.5

The AMPA-evoked responses are present in some ganglion cells at E6 with the majority responding at E8 and later times.
Ganglion cells responding to AMPA during embryogenesis

% of cells responding

<table>
<thead>
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<th>Stage</th>
<th>% of Cells Responding</th>
<th>n</th>
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<td>11</td>
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<td>E14</td>
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<td>18</td>
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Figure 4.6

A) Current-voltage relation of the AMPA-evoked current in a retinal ganglion cell at E12.

Left-hand panel: The peak AMPA current plotted as a function of membrane potential. Bath application of 100 μM AMPA evoked inward currents at holding potentials negative to -15 mV in this ganglion cell. The mean reversal potential at E12 was -12±5.3 mV (n=4). The line is a least squares fit to the data.
Right-hand panel: Individual AMPA-evoked responses from the same cell as in the left-hand panel. The holding potential is indicated next to each trace and the duration of the drug application is shown by the bars above.

B) The AMPA-evoked current is blocked by CNQX.

Left-hand panel: bath application of 100 μM AMPA produced an inward current in this E8 cell.
Centre panel: co-application of the glutamate antagonist CNQX (50 μM) reduced the current response to AMPA by 93%.
Right-hand panel: after a 1 minute wash in Ringer's solution the response to AMPA recovered.
Application of AMPA evoked a membrane current in 93% of the cells (n=14) at E12 and a similarly high proportion of cells responded at E10, 12 and 14. The AMPA current voltage relation was linear with an average reversal potential of -12.0±5.3 mV (n=4) (left-hand panel, figure 4.6A). Individual AMPA-evoked currents at different holding potentials are shown in the right-hand panel of figure 4.6A. Some desensitisation of the current was evident in some cells. Responses to AMPA were blocked by co-application of CNQX. Figure 4.6B shows an example of inhibition of the AMPA-evoked current by CNQX in a ganglion cell at E8. The current recovered after wash-out in Ringer's solution.

4.5 GABA receptor mediated currents in embryonic ganglion cells

GABA is a major inhibitory neurotransmitter in the adult retina. In Ca²⁺-imaging experiments it has been shown that GABA receptors appear very early (E3) in chick retinal development (Yamashita and Fukuda, 1993). During retinal development GABA has been reported to have a depolarising effect (Wu et al., 1992; Cherubini et al., 1991; Yamashita and Fukuda, 1993). In the following experiments the presence of GABA receptors was investigated in retinas between E6 and E14 by bath application of 40 μM of GABA (Tocris). Since binding studies in the rat retina (Schliebs et al., 1986; Schliebs and Rothe, 1988) and Ca²⁺-imaging studies in the chick retina (Yamashita and Fukuda, 1993) suggest GABAₐ receptors to be the predominant GABA receptor during early retinal development, the GABAₐ receptor antagonist bicuculline (50 μM, Tocris) was used in an attempt to block the GABA-evoked responses.
4.5.1 The developmental profile of GABA-mediated responses

Application of GABA evoked a response in 27% of the cells tested at E6 (n=11) (figure 4.7). The fraction of cells responding increased and at E12 all the cells tested (n=10) showed currents. Examples of the GABA-evoked responses at different holding potentials are shown for an E12 ganglion cell in figure 4.8A, right-hand panel). These data show desensitisation or possibly a reduction in current through an accumulation of Cl'-ions near the intracellular face of the membrane (Bormann, 1989). The IV-relation for the GABA current is plotted in the left-hand panel of the same figure. The cell in figure 4.8A showed inward currents at holding potentials negative to -24 mV. The average reversal potential at E12 was -22.2±3.4 mV. The current-voltage relation was linear. Bicuculline, a potent antagonist of the GABA<sub>A</sub> receptor blocked the GABA response (figure 4.8B), this recovered after wash-out of bicuculline.

In patch-clamp experiments the Cl<sup>-</sup>-equilibrium potential is controlled by the Cl<sup>-</sup>-concentrations in the intra- and extracellular solutions. The Cl<sup>-</sup>-equilibrium potential can be calculated using the Nernst equation:

\[ E_{CI} = \frac{RT}{zF} \ln \frac{[Cl^-]_i}{[Cl^-]_o} \]

R: gas constant, 8.315 J K<sup>-1</sup> mol<sup>-1</sup>
T: absolute temperature, 298K
z: valence, -1
F: Faraday's constant, 9.648x10<sup>4</sup> C mol<sup>-1</sup>

With 149.0 mM Cl<sup>-</sup> in the extracellular solution (solution B, table 2.2) and 14.2 mM Cl<sup>-</sup> in the intracellular solution (solution A, table 2.1) is \( E_{CI} \) is -61.4 mV at room temperature (23°C).
Figure 4.7

The percentage of ganglion cells showing GABA-evoked currents as a function of embryonic age.

Responses to GABA (40 μM) were present at the first day tested, E6. By E12-E14, nearly all the cells recorded responded to application of the drug.
Ganglion cells responding to GABA during embryogenesis

% of cells responding

E6  E8  E10  E12  E14
n=11  n=24  n=9  n=13  n=13
Figure 4.8

A) Voltage-dependence of the GABA-evoked current in an E12 ganglion cell.

Left-hand panel: The peak GABA current as a function of the holding potential ($V_h$). The IV-relation is linear and the line is a least squares fit to the data. The currents produced by application of GABA (40 μm) to this cell reversed at -24.1 mV and the average reversal potential at E12 was -22.2±3.4 mV (n=4).

Right hand panel: Individual GABA-evoked currents at different holding potentials (displayed next to each trace). The timing of the application of GABA (40 μM) is indicated by the solid bars. Data from the same cell as in the left-hand panel.

B) Bicuculline blocks the GABA$_A$ receptor mediated current.

Left-hand panel: the GABA-evoked current in an E12 ganglion cell. Centre panel: the current evoked by 40 μM GABA is blocked by co-application of 50 μM bicuculline, a potent GABA$_A$ receptor antagonist.

Right-hand panel: the current response recovered after a 1 minute wash in Ringer’s solution.
**A**

I (pA) vs. $V_h$ (mV)

- $V_h$ (mV): 6 mV
- $V_h$ (mV): -14 mV
- $V_h$ (mV): -39 mV
- $V_h$ (mV): -49 mV
- $V_h$ (mV): -64 mV

30 sec

200 pA

**B**

- GABA
- GABA + bic
- GABA at -45 mV

100 pA

20 sec
The average reversal potential in these experiments is far more positive than the calculated Cl\(^-\)-equilibrium potential, suggesting that the current produced by GABA may not be carried solely by Cl\(^-\)-ions. The possibility that part of the current is carried by gluconate-ions seems unlikely, because of the low permeability of GABA receptor associated Cl\(^-\)-channels to gluconate (Fatima-Shad and Barry, 1993). It is possible that GABA receptor activation releases another transmitter, possibly glutamate or acetylcholine, which then produces an inward current in ganglion cells. These possibilities are considered further in the discussion.

4.6 Glycine-mediated currents in embryonic ganglion cells

Glycine, like GABA, is a major inhibitory transmitter in the retina. In the adult retina 98% amacrine cells are either GABAergic or glycinergic (the proportion depends on the species), and ganglion cells have receptors for both transmitters. In cultured chick retinal neurons GABA\(_A\) and glycine receptors have been shown to be present and linked to Cl\(^-\)-channels (Gleason et al., 1993; Yamashita et al., 1994). However, Ca\(^{2+}\)-imaging experiments have failed to detect any response on application of glycine to the chick retina between E6 and E14 (P. Mobbs - unpublished data).

In the following experiments 100 \(\mu\)M glycine (Sigma) was applied to cells held in the whole-cell mode and the membrane current recorded under voltage-clamp.

4.6.1 The developmental profile of glycine receptor mediated responses

Figure 4.9 shows that early in development, at E6, a small proportion (27%, \(n=15\)) of ganglion cells responded to the application of glycine (figure 4.14).
By E12, all the cells tested responded (n=10) as they did at E14 (E=11). These data show that the temporal pattern of appearance of glycine receptors is very similar to that of GABA receptors (see figure 4.7).

The average reversal potential for the glycine-evoked response was -22.2±3.4 mV (n=3). The right-hand panel of figure 4.10A shows the glycine-evoked currents in an E12 ganglion cell at different holding potentials. Similarly to the responses to GABA seen in figure 4.8 the responses to application of glycine show a strong desensitisation. The current-voltage relation for these currents was linear (figure 4.10A, left-hand panel) and the reversal potential was -23.7 mV. Like the GABA-evoked currents in ganglion cells the reversal potential of the glycine-evoked responses is more positive than the calculated Cl'-equilibrium potential (-61.4 mV). Co-application of 50 μM strychnine abolished the glycine-evoked inward current seen at E12 (figure 4.10B).

4.7 Current density during development

As seen in section 4.3.1 the cell membrane capacitance of ganglion cells increases towards synaptogenesis suggesting an increase in size of the cell's soma and dendritic tree. It is not known yet, whether, once the cell starts to express transmitter receptors, the receptor density in the membrane increases with continuing differentiation of the cell or whether the density of the adult cell is reached early in development. The current density is shown as the peak current amplitude at the same holding potential normalised with respect to the cell's capacitance.

As shown in figure 4.11A the current density of NMDA currents at E8, the first embryonic day that responses to NMDA were observed in ganglion cells, was on average 1.6±0.3 pA/pF (V_h=-25 mV, n=16). Between E8 and E14 there
The percentage of ganglion cells responding to 100 µM glycine increases with embryonic age.

Patch-clamp experiments on embryonic ganglion cells of retinas between E6 and E14 revealed the presence of glycine receptors on the first day tested. The percentage of cells responding to glycine increased from 27% (n=15) at E6 to 100% (n=10) at E12, the beginning of synapse formation.
Ganglion cells responding glycine during embryogenesis

% of cells responding

E6: n=15  E8: n=19  E10: n=9  E12: n=10  E14: n=11
Figure 4.10

A) Voltage-dependence of the glycine-evoked currents in an E12 retinal ganglion cell.

Left-hand panel: The peak currents produced by application of 100 μM glycine are plotted as a function of the holding potential. The trendline is a least squares fit to the data. The reversal potential lies at -23.6 mV. The average reversal potential for cells at E12 was -22±3.4 mV (n=3).

Right-hand panel: The current traces at different holding potentials from the same cell as shown in the left-hand panel.

B) Strychnine blocks the glycine response completely.

Left-hand panel: The current response evoked by 100 μM glycine at a holding potential of -60 mV was abolished by co-application of 50 μM strychnine. The current recovered partially after ca. 45 minutes wash in Ringer’s solution.
were only minor changes observed in the current density, and there was no detectable increase or decrease towards the time at which synaptogenesis begins (E12). AMPA-evoked currents showed a small current density at E6 (1.3±0.6 pA/pF, n=6) compared to the values seen at E8 (4.1±0.9 pA/pF, n=23). Between E8 and E14 there was no significant change in the current density of AMPA receptor mediated current (see figure 4.11B). These data suggest that the number of NMDA and AMPA receptors inserted in the membrane over this period may simply increases in proportion to the expansion of the cell membrane area.

At E6 GABA responses were measured only in one cell at -60 mV resulting in a current density of 0.5 pA/pF. At E8 the current density of GABA receptor mediated currents was high (8.1±3.3 pA/pF, n=4) compared to that at E10 (1.9±0.4 pA/pF). At E12 the current density was 9.1±3.5 pA/pF. The variability in these data may either reflect variations in the GABA receptor density or may simply be due to the small number of cells tested.

Similar to GABA, glycine was applied to only one cell at a holding potential of -60 mV eliciting a current response with a density of 0.8 pA/pF. Figure 4.12B shows that the glycine receptor current density gradually increased between E8 and E14 (0.8±0.3 pA/pF, n=4 and 3.4±0.7 pA/pF, n=13, p=0.06) taking into consideration that the reduction from E12 to E14 is not significant (p=0.40).
A) NMDA current density does not appear to increase with time.

The amplitude of the peak currents produced by application of 50 µM NMDA was normalized to the capacitance of the cell to calculate the current density. The average of all currents obtained at -25 mV holding potential is displayed with the standard error. At E6 none of the cells tested responded to NMDA. Two days later NMDA receptor current density was 1.6±0.3 pA/pF, a value that lies between those recorded during synaptogenesis (2.2±0.3 pA/pF and 1.1±0.3 pA/pF at E12 and E14 respectively).

B) AMPA current density increased significantly between E6 and E8.

The average current density was low early in development (1.3±0.6 pA/pF at E6, V_h=-60 mV), but increased significantly (p=5%) within the following two days to 4.1±0.9 pA/pF. The current densities at E8, E10, E12 and E14 were not significantly different.
A

NMDA currents (pA/pF) at -25 mV

B

AMPA currents (pA/pF) at -60 mV
Figure 4.12

A) GABA receptor current densities were extremely variable in amplitude during development.

At E8 and E12 the current density was 8.4±3.0 pA/pF (n=4) and 9.1±3.5 pA/pF (n=4) respectively. At E10 and E14 the GABA current densities were smaller (1.9±0.4 pA/pF and 4.9±0.7 pA/pF, respectively). The fall in current density between E8 and E10 was significant at the 5% level. \( V_h \) was -60 mV.

B) The glycine-evoked current density increased from E6 to E14.

At \( V_h =-60 \) mV current densities for E6, E8, E10, E12 and E14 were 0.8 pA/pF, 0.8±0.3 pA/pF, 2.8±1.6 pA/pF, 3.3±0.7 pA/pF and 2.2±0.6 pA/pF, respectively.
A  
GABA currents (pA/pF) at -60 mV

B  
Glycine Currents (pA/pF) at -60 mV
4.8. Discussion

4.8.1 The retina as a preparation in which to study the ontogeny of neurotransmitter receptors

In the developing chick retina neurons are born in two phases. During the early phase ganglion, amacrine, type A horizontal, and cone photoreceptor cells are generated while at later stages bipolar, type B horizontal and rod photoreceptor cells are added (Kahn, 1974). Although the times of birth and differentiation for different cell types overlap considerably, the general trend in all vertebrate retinæ is for ganglion cells to be born and differentiate first (40% are born by E3 in the chick; Snow and Robson, 1994), while bipolar cells tend to be last (Sidman, 1961; Webster 1983; Prada et al., 1991; Watanabe et al., 1991). The experiments described here were carried out between E6, when circa 75% of ganglion cells have undergone their final mitosis (Snow and Robson, 1994), and E14, two days after synaptogenesis has begun (Sheffield and Fischman, 1970; Hughes and La Velle, 1974). Müller cells, the predominant glial cell type found in the chick retina (Schnitzer 1987) are born and differentiate later on (Bolz and Wolburg, 1992). Recently, Prada et al. (1998) have found a small population of astrocyte-like glial cells that has never been described in the chick retina before and nothing is known concerning their birth dates. Since ganglion cells are the first neurons in the retina to be born and to differentiate, it is likely that they are also the first to express neurotransmitter-operated channels. Whether these channels are expressed transitionaly or are typical of their adult phenotype, remains to be shown.

Most of what we know of the sequence of appearance of transmitter-operated channels in the retina has developed from studies that have employed optical
measurements of $[\text{Ca}^{2+}]_j$ to determine when they are first present. Optical techniques have the disadvantage that they have limited sensitivity, and unless combined with confocal microscopy or used with retinal slices, have poor spatial resolution. Here I have shown that the whole-cell patch-clamp technique, which in theory can detect the presence of small numbers of channels, can be used to determine both when and where certain transmitter-operated channels appear. I have also shown that patch clamp recordings can be made from chick ganglion cells from early times. That this is possible from times long before synapses form suggests that it may be a useful preparation in which to study the development of transmission via synapses and gap junctions.

4.8.2 The role of AMPA and NMDA receptors in the development of the retina and brain

Glutamate mediates fast excitatory neurotransmission by activating cation-selective channels with distinct gating kinetics, ion permeabilities, conductances and pharmacological properties depending on the type of glutamate receptor subunits that form the channel. Still all ionotropic glutamate receptor subunits belong to a common gene family (for reviews see Nakanishi 1992; Seeburg 1993; and Bettler and Mulle, 1995). To determine when glutamate receptors first appear in retinal ganglion cells they were patch-clamped in whole-cell mode and their membrane currents recorded during application of NMDA or AMPA (50 and 100 $\mu$M, respectively) at different holding potentials. Responses to kainate were not investigated.

The results show that at E6 AMPA receptors were expressed and functional in some ganglion cells (27%), while none responded to NMDA (see figures 4.3 and 4.5). Two days later, both receptor subtypes were present in the
majority of ganglion cells tested and no significant change in the fraction of cells responding to glutamate receptor agonists occurred up until E14 the latest time at which experiments were carried out. Thus AMPA-evoked currents can be recorded from ganglion cells before NMDA produces a response, a result consistent with that obtained in Ca$^{2+}$-imaging experiments. Allcorn (PhD thesis 1995) showed that rises in [Ca$^{2+}$], could first be evoked by application of kainate at E6 and increased towards synaptogenesis (see figure 4.13) and that kainate was acting at AMPA receptors since the responses could be suppressed with the AMPA-specific antagonists CNQX or NBQX. She was unable to detect any change in [Ca$^{2+}$] in response to application of NMDA prior to E8, but showed that subsequently the response to this agonist rose up until E13 the latest time at which experiments were performed (see Figure 4.13). Sugioka et al. (1998) have carried out experiments similar to those of Allcorn and have suggested that kainate receptors play a role in the wave of cell death, that occurs in the GCL and the inner part of the INL between E9 and E16, because the rise in [Ca$^{2+}$], produced by stimulation of these receptors in their experiments reaches a maximum at E9-10. The reasons for the early peak response to kainate application in Sugioka’s experiments remain unclear. A causal role for these receptors in the death of these cells has yet to be demonstrated.

The results presented in this thesis show that the current density of the NMDA and AMPA response (measured as the peak currents evoked by application of the agonists normalised to the cell's capacitance) in ganglion cells reaches a plateau at E8 and does not change significantly up until E14. This is different to the pattern of response obtained using Ca$^{2+}$-imaging experiments where the Ca$^{2+}$-signal increases with time (at least until E13, the last day that was tested (S. Allcorn, PhD thesis 1995). One might expect Ca$^{2+}$-imaging and electrophysiological measurements to produce quite different impressions concerning receptor density. The AMPA and NMDA receptors expressed at different times in development may have different Ca$^{2+}$-permeabilities due to the differences in the subunit composition of the
channels. It has been shown that in the mouse retina the GluR2 subunit, which is responsible for the low Ca\textsuperscript{2+}-permeability of heterologous AMPA receptor channels, is expressed two days later in development (E16) than GluR1 and GluR4 subunits (Zhang et al., 1996). It should be borne in mind that the Ca\textsuperscript{2+}-imaging values are the result of the combined Ca\textsuperscript{2+}-influx into all the cells of the retina, while the electrophysiological responses are from a single cell type. Also, the Ca\textsuperscript{2+}-influx may be amplified to a greater or lesser extent by release from intracellular stores.

Interestingly, the sequence of appearance of glutamate receptor-subtypes in the chick retina is different to that seen in the developing brain. In the developing mammalian hippocampus (Durand et al., 1996; Liao et al., 1995) and optic tectum of the tadpole (Wu et al., 1996) glutamatergic transmission is initially mediated purely by NMDA receptors. A similar sequence has been described for the neocortex (LoTurco et al., 1991) and the somatosensory cortex (Yuste and Katz, 1991). Since NMDA receptors have a much higher affinity for glutamate, about 500 times that of AMPA receptors (Pateneau and Mayer, 1990), they could possibly be activated in the absence of electrical activity and mature synapses simply by background levels of glutamate. However, in the somatosensory cortex of P1-P7 postnatal rats, a time in development, which corresponds to the 'critical period' during which its organisation can undergo plastic changes, synapses formed of NMDA receptors have been shown to be silent, because they are blocked by Mg\textsuperscript{2+} ions. The conversion of such 'silent' synapses into functional ones would require the depolarisation of the cell through activation of other ligand-activated channels or electrical activity.

In the neonatal hippocampus patch clamp experiments have shown NMDA receptors with a reduced voltage-dependency and Mg\textsuperscript{2+}-sensitivity and a higher affinity for glycine when compared to the adult (Ben-Ari et al., 1988, Bowe and Nadler, 1990, Morisset et al., 1990; Kleckner and Dingledine, 1991). These different properties of the receptor channel complex may be
due to a different subunit composition during development different to that of the adult (Monyer et al., 1994). The temporal expression pattern for mRNA coding for NMDAR1 and NMDAR2B from the superficial superior colliculus in neonatal rat showed a low level of NMDAR1 mRNA (less than 50% of the adult) during the first week, while at the same time levels of NMDAR2 mRNA were more than double of those found in the adult (Hofer and Constantine-Paton, 1994). The NMDAR2 subunit in heterologous channels is responsible for the channels Mg$^{2+}$-sensitivity (Burnashev et al., 1992). Thus NMDA receptors have properties at early times in development that should facilitate their operation, GABA may play an important role in depolarising developing neurons that express NMDA receptors. At early times in development GABA has a depolarising effect (see below) and mediates the so-called 'giant depolarising potentials' (GDPs) seen in hippocampal neurons (Ben-Ari et al., 1989; Khazipov et al., 1997; and Leinekugel et al., 1997). GABAergic synapses in the hippocampus are established before glutamatergic ones (Ben-Ari et al., 1989; Hosokawa et al., 1994; Durand et al., 1996) and GABA$_A$ receptor-mediated depolarisation potentiates Ca$^{2+}$-influx through NMDA channels in neonatal CA3 neurons (Leinekugel et al., 1997). Patch-clamp recordings have shown that GDPs are driven by the synergistic activation of GABA$_A$ and NMDA receptors in pyramidal cells and interneurons (Khazipov et al., 1997; Leinekugel et al., 1997). Confocal microscopy and Ca$^{2+}$-imaging have shown that these GDP's are associated with Ca$^{2+}$-oscillations that are mediated via voltage gated Ca$^{2+}$-channels (Leinekugel et al., 1995).

LoTurco et al. (1995) have shown that in the developing rat neocortex GABA and glutamate depolarise cells in the ventricular zone and that the glutamate receptors involved are of the AMPA preferring type (LoTurco et al., 1995). Thus, it is possible that in the brain AMPA receptors are expressed by proliferating cells, while NMDA receptors are important in the synaptic rearrangement of the initial contacts of differentiated cells. In the retina this is clearly not the case since neither AMPA-evoked currents nor changes in
[Ca$^{2+}$], can be detected until E6, by which time much of retinal cell proliferation has already occurred. Studies using the cobalt-labelling technique developed by Pruss et al. (1991) to label cells that express AMPA receptors show that while differentiated cells in the retina are strongly labelled because they express Ca$^{2+}$-permeable AMPA receptors, cells in the ventricular zone are not (Allcorn et al., 1997).

It is possible that the AMPA receptors found in ganglion cells and other retinal neurons at early times are important in the regulation of processes such as neurite extension prior to the development of spontaneous electrical activity. These receptors are well suited for this role, because unlike NMDA receptors that require depolarisation to remove their Mg$^{2+}$-blockade, activation of Ca$^{2+}$-permeable AMPA is possible at hyperpolarised potentials and will result in Ca$^{2+}$-influx. This argument is supported by the fact that early in development of the retina high levels of extracellular glutamate are present (see chapter 3).

4.8.3 The reversal potential of AMPA and NMDA-evoked responses

The reversal potential of NMDA currents in ganglion cells was on average 0.5±3.5 mV (n=5) and linear over the voltage range examined. This is similar to the value obtained for the NMDA receptor in other cells (Jonas and Sakmann, 1992). The reversal potential of the AMPA current was -8±5.3 mV (n=4). This result is similar to the findings of Chiba and Saito (1995) for the AMPA and kainate-evoked responses of newt retinal cells, AMPA receptors of rabbit starburst amacrine cells of the rabbit retina (Zhou and Fain, 1995) and many neurons in the brain.
4.8.4 The role of GABA and glycine receptors early in the development of the retina and the brain

GABA and glycine, the most common inhibitory transmitters in the vertebrate CNS, have been localised at synapses between amacrine and ganglion cells (Miller et al., 1981; Glickman et al. 1982; Bolz et al., 1985a; Bolz et al., 1985b; Muller and Marc, 1990) In the adult retina GABA receptors have been localised in all cell types of the retina: ganglion cells (Ishida and Cohen, 1988; Tauck et al, 1988), amacrine cells (Yamashita et al., 1994) including interplexiform cells (Gustincich et al., 1997), bipolar cells (Gillette and Dacheux, 1995; Qian and Dowling, 1995), horizontal cells (Stockton and Slaughter, 1991; Cammack and Schwartz, 1993; Blanco and de la Ville 1996) and photoreceptors. Further studies with antibodies revealed GABA receptors on cone terminals as well as on cone bipolar dendrites (Vardi et al. 1992).

The results of this chapter show, that GABA receptors are present in ganglion cells at E6 through to E14. The fraction of ganglion cells responding to GABA increased until E12, when the all cells tested responded (n=10). The fraction of cells responding increases sharply, from 35% to 100% (n=9 and 10, respectively), between E10 and E12. The appearance of glycine receptors followed the same pattern as that of GABA receptors.

In accordance to the results obtained from chick retina, in the mammalian retina Rörig and Grantyn have shown GABA and glycine receptors to be present on ganglion cells of the embryonic mouse retina at times well before synapses start to form (1994). Similarly in the brain GABA and glycine receptors are present from early times in neocortical and hippocampal development (Ben-Ari et al., 1989; Malosio et al., 1991; Sato et al., 1992;
Hosokowa et al., 1994 and Durand et al., 1996). The $\text{GABA}_A$ receptor found at these early times may be located in progenitor cells, since they are situated in the ventricular zone of the retina (P. Mobbs, unpublished data) and are also expressed by cells of the neural plate (A. Randall, pers. com.). It has been shown using $\text{Ca}^{2+}$-imaging of whole-mount retina that $\text{GABA}_A$ receptors are present in the embryonic chick from E3 onwards (Yamashita and Fukuda, 1993).

The fact that in my experiments fewer cells respond to GABA early in development than at E14 does not necessarily imply that GABA receptors do not have important roles at early times. Indeed $\text{Ca}^{2+}$-imaging experiments (S. Allcorn, PhD thesis 1995) on whole-mount retinae showed robust GABA-evoked $\text{Ca}^{2+}$-responses from E4 through to E12. GABA-evoked $\text{Ca}^{2+}$-responses peaked at E8 and were absent at E13, probably because the GABA response becomes shunting or hyperpolarising at this time. Similar results were obtained by Yamashita and Fukuda (1993) who report GABA responses from E3 onwards with the largest responses occurring at E9. In contrast the results of the whole cell patch clamp experiments reported here show the largest number of ganglion cells responded with higher current amplitudes at later times. Since the responses in the optical recordings reflect the responses of all the cells present in the tissue the difference between imaging-experiments and this study may simply reflect the expression of GABA receptors in cells other than ganglion cells at early times (for example cells in the ventricular zone). Changes in the $\text{Cl}^-$-reversal potential in differentiated cells may explain the reduction in the GABA-evoked $\text{Ca}^{2+}$-signal at later times.

The excitatory action of GABA and glycine at early times in development is a general feature of the developing nervous system and has been shown in the development of chick spinal cord neurons (Obata et al., 1978) and in the
developing brain (for review see Cherubini et al., 1991 and Ben-Ari et al., 1997). It results from the high intracellular Cl⁻-concentration in immature neurons (Owens et al., 1996) forcing Cl⁻-ions out through these receptor channels at membrane voltages near the resting potential. The GABA and glycine receptors of ganglion cells have been shown to be Cl⁻-selective in several species (Tauck et al., 1988; Ishida and Cohen, 1988; Cohen et al., 1989). In whole-cell patch-clamp experiments the intracellular and extracellular ion concentrations are set by the solution in the patch pipette. With the solutions used in the experiments described in this chapter Cl⁻-concentrations inside and outside cells were similar to those found in the adult nervous system giving a reversal potential of -60 mV for a Cl⁻-specific channel. Surprisingly, at E12 the average reversal potential for the GABA-evoked current was -18.2±3.4 mV (n=4). The reversal potential of the glycine responses obtained was similar. Gluconate was used in the patch pipette solutions employed, and but the permeability of GABA and glycine channels for gluconate is low (Fatima-Shad and Barry, 1993) and thus, it is unlikely that a gluconate current contributes to the reversal potential.

A possible explanation for a positive shift of the GABA and glycine reversal potential is, that the application of GABA leads to the release of some other transmitter. This could be glutamate or ACh released from amacrine and/or bipolar cells, since both of these cell types possess GABA and glycine receptors (Yamashita et al., 1994; Gillete and Dacheux, 1995; Qian and Dowling, 1995; Gustincich et al., 1997). Unfortunately, the use of Ca²⁺-free HEPES buffered Ringer led to a rapid run down of the retina and cell death making it impossible to assess the contribution of the Ca²⁺-evoked release of transmitter to the responses recorded from ganglion cells. Attempts were also made to isolate the recorded ganglion cell soma from the retina after having entered whole-cell mode, but this proved to be impossible.
GABA receptors have been shown to be present in the embryonic chick retina from E3 onwards (Yamashita and Fukuda, 1993) and to be responsible for Giant Depolarising Potentials involved in Ca\(^{2+}\)-oscillations in the developing brain (see discussion of the glutamate receptor appearance 3.10.2.) (Ben-Ari et al., 1989; Leinekugel et al., 1997). The receptors present on chick retinal ganglion cells during development are likely to be GABA\(_A\) receptors, since their responses are largely blocked by the GABA\(_A\) antagonist bicuculline. It is possible that activation of GABA receptors plays a role similar to that in the developing brain. On the other hand, the amount of GABA measured in the embryonic chick retina at early times were extremely low. However, GABA receptors may be activated by other substances like glycine and taurine, that are present in the retina at higher concentrations (own observations). The small component of the GABA-evoked response that remained in bicuculline may possibly reflect the presence of other GABA receptor subtypes.

4.9 Further experiments

After these electrophysiological experiments were done it was found that embryonic retina can be maintained for long periods in Krebs' solution supplied with high Mg\(^{2+}\) and low Ca\(^{2+}\)-concentration. It would be interesting to repeat and extend some the experiments described above using this solution to block Ca\(^{2+}\)-influx and thus vesicular release. Alternatively, it may be possible to record from ganglion cells after enzymatic dissociation eliminating any possible contribution of other cells to the recorded response.
5.1 Introduction

Gap junction channels are a major pathway for intercellular communication in the CNS. In the adult retina, gap junctional communication between neurons optimises signal detection and processing to suit the prevailing levels of illumination. During development, extensive dye-coupling of neurons has been observed in the rat neocortex (Peinado et al., 1993) and in the mammalian retina (Penn et al., 1994). It has been shown that the extent and pattern of coupling changes during the period that neurons migrate and differentiate within the CNS (Catsicas et al., 1998; Kandler and Katz, 1998a). Such gap junctional communication may play an important role in the synchronisation of the waves of spontaneous activity seen in several regions of the developing nervous system (Yuste et al., 1992; Penn et al., 1994; Peinado et al., 1993; Yuste et al., 1995; Kandler and Katz, 1998b). The aim of the experiments in this chapter was to reveal the extent of gap junctional coupling of ganglion cells during early development of the embryonic chick retina.

5.2. Methods

The pattern of gap junctional coupling of ganglion cells was investigated by whole-cell patch-clamping using pipettes filled with a mixture of fluorescein-dextran (MW 10 KD, 32 mg ml\(^{-1}\)) and Neurobiotin (MW 323 D, 5 mg ml\(^{-1}\)) in solution A (see table 2.2). The low molecular weight Neurobiotin is free to diffuse through gap junctions from the recorded neuron into any cells coupled
to it, while the high molecular weight fluorescein-dextran remains trapped. After histochemical treatment to label the Neurobiotin with a fluorescent marker, the tissue was examined by confocal microscopy. Further details of these techniques are given in section 2.4. In the images shown in this thesis, cells filled with both Neurobiotin and fluorescein-dextran are shown in white, while those filled only with Neurobiotin are shown in bright blue and the antibody staining for Cx 32 in figure 5.12 in bright red.

The images presented in this chapter are stacks of confocal optical sections (ca 1 μm apart) obtained by focusing down from the GCL through the IPL and the INL. These optical sections have been superimposed to produce a two dimensional image in the plane of the retina (termed a projection). The stacks of sections were rotated through 90° effectively giving a two dimensional side-view of the retina with the GCL at the top and the INL at the bottom of the picture (termed a rotation). The area of ganglion cell somata and dendritic fields were measured using NIH Image 1.62b7.

5.3. Results

This chapter describes the morphology of chick ganglion cells and the extent of their coupling to other cells at different times during development. More than 150 cells were whole-cell patch-clamped and filled with dye. The illustrations are representative of the results obtained.

5.3.1 The morphology of ganglion cells during development

E5

Figure 5.1A shows an example of an E5 ganglion cell. At this time typically there are no dendrites and the axon is either in the process of growing along the AFL towards the choroid fissure and the optic nerve or has entered the
optic nerve already. The axon of the cell shown in 5.1 A ended in a growth cone within the AFL as shown in figure 5.1 B. Ganglion cell growth cones are characterised by a thickening of the axon with several spiny extrusions. The cell shown was not coupled to any others, however, this was not always the case at E5 (see section 5.3.2).

Dye was applied to the crushed end of the optic nerve to retrogradely label ganglion cells via their axon to give an overview of the morphology of ganglion cells at this age (figure 5.1 C). The majority of ganglion cells have a morphology similar to the example shown in 5.1A but in some cases short processes extend from the soma (thin arrows). The axons of ganglion cells are not conspicuously bundled as they are later on.

**E7**

By E7, most ganglion cells have developed a small irregularly branched dendritic tree with a few primary dendrites. The examples shown are typical of the range of geometries and coupling seen at this time. The cell shown in figure 5.2 A is coupled to two others (thin arrows), which are located in the same plane as the injected neuron. As seen in the rotation of the confocal stack (figure 5.2 B) the dendrites of the injected neuron project towards the outer retina and lie amongst the cell bodies and processes of other neurons. At this time, there is no obvious division of the retina into nuclear and plexiform layers. Thus, some of the cells coupled to the injected ganglion cell may be ganglion cells and others neurons of the future INL. Three further examples of E7 ganglion cells are shown in figures 5.2 C-F. In one case (figure 5.2 C) the injected cell has very few processes and is coupled to five other cells: four located near its soma and one at some distance along the axon. In the corresponding rotation shown in figure 5.2 D it can be seen that some of the coupled neurons have “trailing processes” (arrowed) that extend towards the outer retina as have been described for migrating cells (Snow and Robson, 1995; Watanabe et al, 1991). Similarly to the cell in C, the
Figure 5.1

Ganglion cell morphology and dye-coupling at E5.

A) The injected ganglion cell can be identified by its axon (open arrow). Most ganglion cells lack dendrites at this age. This cell is not dye-coupled to any others.

B) The growth cone of the outgrowing axon (open arrow) of the cell in A.

C) An overview of ganglion cell morphology at E5 produced by backfilling the optic nerve with dye. A small number of cells have short processes (thin arrows). Axons tend not to be bundled as they are later on.

In A and B scale bars represent 10 µm, in C 20 µm.
Ganglion cell morphology and dye-coupling at E7.

A) A ganglion cell of high dendritic complexity for E7. Its axon has grown in the opposite direction before turning towards the optic nerve. This cell is dye-coupled to two others (thin arrows).

B) Rotation of the confocal stack shown in A. A single process reaches right into the depth of the retina into the future INL, while the other dendrites ramify in the region where the IPL will develop. The axon loops down amongst other cell bodies and then turns to the vitread surface of the retina.

C) A ganglion cell almost devoid of dendritic processes. It is coupled to 4 of its neighbours via its soma forming contacts to their dendrites (thin arrow), while a fifth cell is coupled via its dendrite (dashed arrow) to the axon of the injected neuron.

D) Rotation of the confocal stack of the cell in C reveals the thin trailing processes of the coupled cells (open-headed arrows). The axon of this cell is labelled with an open arrow.

E) A ganglion cell with two dendritic processes, one of which (thin arrow) contacts the soma of a neighbouring ganglion cell (filled arrow indicates this cell’s axon) and presumably represents the site at which these two cells are coupled.

F) A ganglion cell coupled to 7 other cells (well above the average number for this age: 1.3±0.4, n=30). While most of these cells are coupled dendrosomatically (thin arrow), one of them (dotted arrow) is coupled via its dendrites to the axon of the injected cell.

The open arrows indicate the axon of the injected ganglion cell. Scale bars represent 20 μm.
injected ganglion cell shown in figure 5.2 E is coupled dendro-somatically (thin arrow) and via its axon to the dendrite of another cell (dashed arrow). The ganglion cell in figure 5.2 F is coupled to one other via a single stout dendrite (white filled arrow indicates the axon of the coupled cell).

**E9**

By E9 the dendritic tree of ganglion cells have developed much further and it begins to be possible to discern several subtypes. The examples of E9 ganglion cells shown in figure 5.3 A, B and 5.4 A, B are similar cells to Group Ib and II described by Thanos et al. (1992) in posthatch chick retinae. The cell in figure 5.3 A is similar to the Group Ib subtype in that it has a small soma (71 μm²) and a small sparsely branched dendritic tree with beaded dendrites (dendritic field area 8547 μm²). It is coupled to other cells, which, as can be seen in the rotation in figure 5.3 B, are located in both, the GCL and the INL. The brightest and largest soma in this image may be that of a displaced ganglion cell.

The cell shown in figure 5.4 A is similar to Thanos et al.’s (1992) Group II cells in that it has a medium sized soma (229 μm²) and a small (5847 μm²) complex dendritic tree. This morphology is similar to that of β-ganglion cells described in the mammalian retina (see for example Boycott and Wässle, 1974). This cell was coupled to 20 others, some located in the GCL and others in the INL (see the rotation in figure 5.4 B).

**E11**

By E11, there has been a considerable increase in the area of ganglion cell dendritic trees, some of them approaching their adult dimensions, while others remain relatively immature in their morphology. The extent of coupling has also increased significantly (see section 5.3.2). Bistratified ganglion cells first become apparent at E11, an example of such a cell is shown for E14 in figure 5.7 A, B.
Ganglion cell morphology and dye-coupling at E9

A) This example of a ganglion cell at E9 is characterised by a small soma (area 71 µm²) and a small dendritic field (area 8547 µm²). The dendrites are beaded and sparsely branched. This cell is coupled to 18 others. The morphology of this neuron is similar to that described for Group Ib ganglion cells by Thanos et al. (1992).

B) The rotation of the cell shown in A. Most coupled cells are located above the dendritic tree in the GCL and only a few below it in the IPL. One brightly labelled, big soma in the IPL (thin arrow) may be that of a displaced ganglion cell.

The open arrows indicate the axon of the injected ganglion cell. Scale bar represents 20 µm.
Figure 5.4

Ganglion cell morphology and dye-coupling at E9

A) A type Group II (β-like) ganglion cell injected at E9. This cell has a small, complex dendritic tree (area 5847 \( \mu m^2 \)) and a medium sized soma (area 221 \( \mu m^2 \)). It is coupled to 20 other cells.

B) Rotation of the confocal stack of the cell in A shows that the dye-coupled cells are located above and below the dendritic tree of the injected cell.

The open arrows indicate the axon of the injected ganglion cell. Scale bar represents 20 \( \mu m \).
Figure 5.5 A shows one of the more “mature” cells with a medium to large soma (255 μm²) and a complex, asymmetric dendritic field with an area of 34453 μm². Cells of this morphology are similar to the Group V neurons described by Thanos et al. (1992) and the α-ganglion cells of the mammalian retina (see for example Boycott and Wässle, 1974). This cell was extensively coupled (to 71 others) to cells in the GCL and INL, two of which have processes that span the thickness of the retina (see rotation in figure 5.5 B). The coupled cells vary in the intensity with which they are labelled by Neurobiotin. The intensity is not directly related to the distance at which the cells lie from the soma of the injected ganglion cell. It is possible that the weaker labelled cells are coupled indirectly via gap junctions to cells that form primary contacts with the injected neuron. Another example of an E11 ganglion cell is shown in figure 5.6 A, B. This cell is similar to the one shown in figure 5.4 A, B for E9 a Group II cell according to Thanos et al.’s (1992) classification criteria. Both cells have small to medium sized somata and small dendritic fields (185 μm² and 4826 μm² respectively at E11). However, the dendrites of the older cell are more clearly separated from the nuclear layers (GCL and INL) than those of the younger one at E9.

E14

By E14 the different subtypes of ganglion cells present in the retina become more distinct. In addition to the Group Ib, II and V (the first two groups are equivalent to β-ganglion cells and the latter to α-ganglion cells) two further ganglion cell subtypes can be identified. The first is shown in figure 5.6 A, B, it has a large soma (360 μm²) and a complex, bushy dendritic tree (area: 19625 μm²). In the rotation of the confocal stack, shown in figure 5.7 B, the bistratification of the dendritic tree is clearly visible. Although the area of the soma is at the higher end of the range described by Thanos et al. (1992) for their Group III cells, this neuron is otherwise very similar to the cells of this group. Thanos et al.’s Group III neurons are also similar to the β-type ganglion cells found in mammalian retinae (Boycott and Wässle, 1974).
Ganglion cell morphology and dye-coupling at E11

A) An example of a Group V (α-like) ganglion cell at E11. The cell has a large, complex, asymmetric dendritic tree (34453 μm²) and a medium to large soma (255 μm²). It is coupled to large number of other ganglion and amacrine cells. Some of the cells containing Neurobiotin do not appear to be directly coupled to the injected cell, but rather appear to have become filled through contacts with cells that are directly coupled.

B) A rotation of the projection shown in A. Two of the Neurobiotin filled cells (thin arrows) are located in the INL and have processes that span the thickness of the retina.

The open arrows indicate the axon of the injected cell. The scale bar represents 50 μm.
Ganglion cell morphology and dye-coupling at E11

A) An example of a group Ib (β-like) ganglion cell injected at E11. This cell type is characterised by a small to medium sized soma and a small complex dendritic tree (areas 185 μm² and 4816 μm², respectively, for the neuron shown).

B) Rotation of the confocal stack shown in the projection in A. While the injected ganglion cell appears to be coupled to ganglion cells of the same type and to other cells in the GCL, it is also coupled to putative amacrine cells in the INL.

The axon of this cell lies within those sections omitted from the confocal stack. The scale bar represents 20 μm.
Figure 5.7

Ganglion cell morphology and dye-coupling at E14

A) A Group III (β-like) ganglion cell with a large soma (area 360 μm²) and small to medium sized bistratified, bushy dendritic tree (area 19625 μm²). This cell is coupled to 4 other neurons two of which are localised in the INL.

B) Rotation of the confocal stack shows the stratification of this cell’s dendritic tree. The thin arrows point to the coupled cells in the INL and the dashed arrows to the ones in the GCL.

The axon of this cell lies within sections omitted from the confocal stack. The scale bar represents 20 μm.
Ganglion cell morphology and dye-coupling at E14

A) An example of a Group VII (γ-like) ganglion cell injected at E14. This cell type has long sparsely, branched dendrites that extend over a large area (in this case 50732 μm²). The somata are irregular in shape and small in comparison to the dendritic field area (soma area of the cell shown is 265 μm²). This neuron is coupled to two others.

B) Rotation of the confocal stack shown in A. The cell bodies of the coupled cells lie centrally in the INL and have processes that span the retina.

The scale bar represents 20 μm. The axon of this cell has been broken in the AFL (open arrow).
The second cell type identified at E14, shown in figure 5.8, has long sparsely branched dendrites (dendritic area 50732 pm²) and an irregularly shaped cell body (265 pm²), the exact appearance of which varies from cell to cell. In Thanos et al.'s classification these cells are termed Group VII and they are similar to the γ-type cells of the mammalian retina (for example Boycott and Wässle, 1974).

5.3.2 Increase in soma size and dendritic field size during development

In order to compare the dimensions of the injected ganglion cells shown in this chapter with the cells of the only other study by Thanos et al. (1992), the area of the soma and the dendritic field of 131 cells was measured using NIH Image version 6.4.2. Figure 5.9 shows the distribution of ganglion cell soma and dendritic field size at different times in development. At E5 (panel A) ganglion cells were characterised by small somata with an area between 68-152 μm² (n=17) and an average of 106±6.4 μm². At this time that the first processes emerge from the soma and form small polymorphic dendritic fields of area 0-5372 μm² (662±302 μm²). By E7 (figure 5.9, panel B), the average soma size has increased to 123±6.1 μm² (range 75-199 μm²; n=37) and the mean dendritic field area to 901±120 μm² (range 0-2952 μm²). At E9 (figure 5.9, panel C), the mean dendritic field area was 5101±6.4 μm² (range 494-19282 μm²; n=22). Despite the large increase in dendritic field size, soma size only slightly increased (131±8.5 μm², range 71-229 μm²). At E11 (figure 5.9, panel D), the dendritic field area has more than doubled (10312±1653 μm², range is 911-40482 μm²), while the soma size is roughly the same as at E9 (140±9.1 μm²; range 62-273 μm² n=23). By E14 (figure 5.9, panel E), a major increase in soma size (256±15.6 μm²) and dendritic field size (12381±2814 μm²) has occurred (n=23).
Figure 5.9

Graph showing the range of the dendritic field and somata areas of ganglion cells at E5, E7, E9, E14.

A) The distribution of ganglion cell dendritic field area and soma area at E5 (662±302 \( \mu \text{m}^2 \) and 106±6.4 \( \mu \text{m}^2 \), respectively, n=17).

B) The dimensions of ganglion cell dendritic fields and somata at E7 (901±120 \( \mu \text{m}^2 \) and 123±6.1 \( \mu \text{m}^2 \), respectively; n=37).

C) By E9 the mean area of the somata (131±8.5 \( \mu \text{m}^2 \)) is slightly larger and dendritic field area has increased five fold (5101±6.4 \( \mu \text{m}^2 \), n=22).

D) By E11 soma area has undergone a further small increase (145±8.3 \( \mu \text{m}^2 \)), and again, there has been a significant increase in the dendritic field area (10312±1653 \( \mu \text{m}^2 \), n=32).

E) By E14 both soma area (256±15.6 \( \mu \text{m}^2 \)) and dendritic field area (12381±2814 \( \mu \text{m}^2 \), n=23) have increased significantly.

The inset shows the mean area of ganglion cell somata and dendritic fields as a function of time.
Increase in soma and dendritic field area during development

The average soma and dendritic field area

area of the soma

(\mu m^2)

dendritic field area

(\mu m^2\times10^3)

A

E5

B

E7

C

E9

D

E11

E

E14
5.3.3 Increase in the degree of coupling of the injected ganglion cells towards synaptogenesis

Between E5 and E14 more than 150 ganglion cells were injected to determine their patterns of gap junctional coupling. From the confocal microscope images obtained, the number of cells coupled to a single ganglion cell was determined. Coupled cells, where possible, were classified on the basis of the position of their somata.

Figure 5.10 shows that at E5 and E7 cells were coupled on average to just one other cell (1.07±0.27 and 1.33±0.37, respectively). At E5, 6 out of 15 cells were not coupled to any other cell, and at E7, 15 out of 30 ganglion cells showed no trace of coupling. At E11, the number of cells coupled to the injected ganglion cell was significantly higher when compared to E7 13.12±2.34 (n=50; p<0.001). By E14 the number of coupled cells had fallen significantly to 2.93±0.91 (n=29; p<0.01). Thus, gap junctional coupling of ganglion cells to other cells increases during development reaching a peak at the onset of synaptogenesis and subsequently declines.

From rotations of the confocal image stack, it is clear that at early times ganglion cells are coupled to other nearby cells that lie in the most vitread layer of cell bodies. While at E7 there is no obvious separation of the cell bodies of GCL neurons from those of the INL, it is clear that some coupled cells lie vitread of the dendrites of ganglion cells and others on the other, outer, side of them. Thus, at E7 about 15% of the coupled cells appear to belong to a population of cells in the future INL. With time the proportion of coupled cells in the INL increases reaching 45% by E14. This change in location of the coupled cells is shown in figure 5.11.

Throughout the entire period examined, some ganglion cells were found that were coupled to neurons with a bipolar morphology that spanned the entire thickness of the retina and whose somata were localised deeper in the INL than most of the other coupled cells (see figure 5.4 B and 5.5 B). On average
Figure 5.10

The number of cells coupled to a single ganglion cell during embryogenesis.

At E5 and E7 ganglion cells were coupled on average to one other cell (1.07±0.27 and 1.33±0.37, respectively). A marked increase in the number of coupled cells was seen at E9 and the highest degree of coupling was observed at E11 (13.12±2.34; p<0.001 comparing E7 to E11), just before synapse formation begins. Two days into synaptogenesis, the extent of coupling had decreased to 2.93±0.91 (p<0.01).
Number of coupled cells to ganglion cells during development

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of Coupled Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>E5</td>
<td>15</td>
</tr>
<tr>
<td>E7</td>
<td>30</td>
</tr>
<tr>
<td>E9</td>
<td>24</td>
</tr>
<tr>
<td>E11</td>
<td>50</td>
</tr>
<tr>
<td>E14</td>
<td>29</td>
</tr>
</tbody>
</table>
Figure 5.11

Somatic location of neurons coupled to ganglion cells

Cells coupled to ganglion cells were found in both the GCL and the INL. The proportion of coupled cells located in the GCL decreased from 73% at E57 to 32% at E14. The opposite trend was seen in the INL, in which the percentage increased from 15% at E7 to 45% at E14.

Since only few of the cells have developed dendrites at E5 it is impossible to allocate the coupled cells to either the GCL or INL. All of the coupled neurons at E5 are located at the same depth in the retina as the injected ganglion cells and, for the purpose of this figure, are shown as lying in the GCL. Those cells that span the retina are not shown in this histogram.
Somatic location of neurons coupled to ganglion cells during development

- % in GCL
- % in INL

% of coupled cells

- n=15
- n=30
- n=24
- n=49
- n=29

E5  E7  E9  E11  E14
there was less than one such cell associated with each ganglion cell filled. The identity of these cells is unclear, some maybe neurons in the process of migration from the ventricular zone to more vitread positions.

5.3.4 Coupling sites

Since Cx 32 is believed to be one of the connexins responsible for gap junctional coupling between neurons (Becker et al., in preparation), its distribution within the GCL was investigated to determine the location of potential sites of coupling. Cx 32 plaques are found in the membranes of the somata, dendrites and axons of cells in the GCL suggesting that gap junctions can form between any part of a ganglion cell and its neighbours. Antibodies to Cx 32 were used to label preparations in which ganglion cells had been injected with the two dyes. This was successful in only a few cases but revealed that Cx 32 plaques appear to be localised at the point of contact between the injected ganglion cell and the cells coupled to it filled with Neurobiotin. Figure 5.12 shows an example of an E11 retina labelled with antibodies against Cx 32. In this case the points of contact between the ganglion cell and the coupled cells appear to be dendro-somatic and are coincident with large plaques of Cx 32 (thin arrows) as could be worked out by comparing the projection and rotation of these cells. To confirm that the point of contact is indeed the location of a gap junction formed of Cx 32, further studies using electron microscopy would be necessary. Investigations of the sites of contact between the dye-injected ganglion cells and the cells to which they are dye-coupled (without antibody staining for connexins), showed that at early times coupling seemed to occur more often between the dendrites of the ganglion cell and the soma of another cell, while later in development such contacts lay between the dendrites of the cells (for example the cells shown in figures 5.3-5.5). Occasionally ganglion cells appeared to be coupled to others cells via their axon (figure 5.2C, E and F).
Figure 5.12

Immunolabelling of Cx 32 in a retina from E11

A) This α-type ganglion cell is coupled to two other cells, which are located in the INL (seen in the rotation). The antibodies against Cx 32 used here are linked to CY5. Gap junctional plaques are shown in red. The scale bar represents 20 μm.

B) With a higher magnification the dendrites of the injected ganglion cell could be followed into the depth of the tissue to the point of contact with the somata of the dye-coupled cells. They were found to be co-localised with plaques labelled for Cx 32 (thin arrows). The scale bar represents 10 μm.
5.3.5 Gap junctional modulators

There are several neurotransmitters which modulate gap junctional communication between neurons. In the adult retina, dopamine has been shown to modulate gap junctional conductance between horizontal cells (Mahon and Mattson, 1996) and the tracer-coupling of All amacrine cells (Mills and Massey, 1995). Dopamine receptors of both subtypes, D1 and D2, are present on ganglion cells (for review see Djamgoz and Wagner, 1992) and dopamine has been found to modulate spontaneous activity in the early embryonic chick retina (Catsicas et al., 1998).

In order to determine whether or not dopamine modulates gap junctions between ganglion cells and other neurons in the embryonic chick retina, dye-injections were carried out at E11 in the presence of both high and low concentrations of dopamine (100 μM and 100 nM, respectively). Neither the high nor the low dose of dopamine had a significant effect on the extent of coupling to ganglion cells or cells located in the INL (figure 5.13). Total coupling fell from 13.0±2.2 in control solution to 10.1±3.0 in 100 nM dopamine, but this decrease was not significant (p>0.5).

Since it was possible that the apparent dye-coupling observed following the injection of Neurobiotin into ganglion cells resulted either from leakage of the dye from the electrode and its accumulation by neighbouring neurons or from some other artefact the following experiment was carried out. Cells were filled in the presence of octanol (2mM), a non-specific blocker of gap junctions (Weingart and Bukauskas, 1998; Martins-Ferreira and Ribeiro, 1995; Toraason et al., 1992; see for review Spray et al., 1985; Spray and Bennett, 1985) in the extracellular solution, and the number of coupled cells compared to that seen in its absence. Octanol effectively abolished coupling in both the INL and GCL, the number of coupled cells in total falling from 13.1±2.3 to 0.4±0.2 in the presence of octanol (p<0.01). This result combined with the observation that coupled cells were not found in preparations, where
Figure 5.13

The effects of known gap junctional modulators, octanol and dopamine, on the number of cells dye-coupled to ganglion cells at E11.

In control solution, ganglion cells were coupled to an average of 13.12±2.20 other cells. Octanol (2mM) significantly decreased coupling (p<0.05) in the GCL and in the INL by 96% and 100%, respectively. High (100 μM) and low (100 nM) doses of dopamine had no significant effect on the extent of coupling.
The effect of gap-junctional modulators

The graph shows the number of coupled cells for different conditions: Control, Octanol, Low-dopamine, and High-dopamine. The x-axis represents different cell layers: Total, GCL, and INL. The y-axis represents the number of coupled cells.

- **Total**: The bar for Control is the highest, followed by Low-dopamine and High-dopamine. The number for Control is n=54, for Low-dopamine n=14, and for High-dopamine n=15.
- **GCL**: The bar for Control is the highest, followed by Low-dopamine and High-dopamine. The number for Control is n=26, for Low-dopamine n=14, and for High-dopamine n=15.
- **INL**: The bar for Control is the highest, followed by Low-dopamine and High-dopamine. The number for Control is n=26, for Low-dopamine n=14, and for High-dopamine n=15.

The diagram includes error bars indicating the variability of the data.
the patch electrode was placed near ganglion cells but not sealed to their membranes nor when seals were obtained but attempts to enter whole-cell mode failed, confirms that the pattern of coupling described in this chapter is not artefactual.

5.4 Discussion

Gap junctions represent a pathway by which retinal neurons can communicate both electrically and metabolically. Gap junctions are present before synapses are formed and may play a crucial role in determining the wiring of the retina. The results obtained here, and in other studies, suggest (for review see Rörig and Sutor, 1996) that during development of the CNS the pattern of coupling between neurons is not a simple progression towards the adult pattern, but rather a sequence of interactions between cells that fulfil different tasks at different times.

5.4.1 Changes in the number of cells coupled to ganglion cells with time

At early times in development (E5-E7), ganglion cells are coupled on average to just one other cell and 40-50% of them are not coupled to any of their neighbours. While there is no evidence concerning the dye-coupling of retinal ventricular zone cells (progenitors, newly differentiated neurons and glia), the results obtained here suggest that, if these cells are extensively coupled in the retina as they are in the cortex (LoTurco and Kriegstein, 1991) then this coupling must disappear when the cells migrate to their final destination.

In the developing cortex, neuroblasts in the ventricular zone are dye-coupled extensively, but following their final mitosis this coupling disappears. LoTurco and Kriegstein (1991) suggest that the loss of coupling is the signal to
terminate mitosis and initiate migration. However, there is as yet no experimental evidence for a causal relationship. Once cortical cells have reached their final location, they again show extensive dye-coupling (Connors et al., 1983; Peinado et al., 1993; Mienville et al., 1994; for review see Rörig and Sutor, 1996).

Recently, Kandler and Katz (1998a) have shown a transient increase in dye-coupling between developing neurons of the visual cortex of the ferret. These findings are similar to the results shown here for the developing chick retina. The experiments in this chapter have shown that the extent of gap junctional coupling of ganglion cells increases until E11 just before synapse formation begins. Three days later, coupling decreases significantly (figure 5.10). In contrast, in the developing ferret retina there is a steady increase in coupling between P1 and P45 (Penn et al., 1994), the coupling pattern moving progressively towards that seen in the adult. The reasons for the difference between chick and ferret retina are not clear, however, it would be interesting to examine the extent of coupling of ganglion cells in embryonic ferret retina to determine whether it is greater at times prior to synapse formation.

Although migrating neurons are thought not to be coupled one to another (see above), the dye-injections into ganglion cells of the embryonic chick retina reveal gap junctional coupling to cells that span the thickness of the retina with their somata located in the central part of the INL. The morphology of these cells suggests that they may be neurons in the process of migration (for comparison see Nishimura et al., 1979; Snow and Robson, 1995). Thus, it is possible that migrating chick ganglion cells may form gap junctions, via the process they extend towards the vitread retina during migration, with cells that have already reached their final location. Such coupling is reminiscent of that seen between deep and superficial pyramidal cells in the developing rat neocortex (Peinado et al., 1993). Although the number of putative migrating cells was small (on average less than one per injected ganglion cell at any stage), such coupling was seen at all ages examined. Peinado et al. (1993;
Yuste et al., 1995) suggest that the cell clusters forming columns of coupled cells in the neocortex serve as a functional unit of synchronised spontaneous activity. The dye-coupling between deep and superficial pyramidal cells disappears after a week and concentric coupled cell clusters start to develop in lamina II/III, similar to the coupling of ganglion cells to other ganglion cells in the retina. The hypothesis that gap junctions are the means of synchronising waves of spontaneous activity before and during the time of circuit formation has been suggested for the retina (Penn et al., 1994; Catsicas et al., 1998) as well as in the brain (Peinado et al., 1993; Yuste et al., 1994; Yuste et al., 1995; for review see Katz 1993 and Kandler and Katz, 1995; Kandler and Katz, 1998a, b).

5.4.3 Ganglion cell subtypes during development

Many ganglion cells filled with dye at E11 and E14 could be allotted to one or other of the subtypes described by Thanos et al. in posthatch chick retinae (1992) (see section 5.3.1). However, even at these late times the dendritic architecture of a number of cells was too immature to enable them to be unambiguously classified. Group I-III ganglion cells could be identified in the embryonic retina both by virtue of their dendritic pattern and their dimensions, while cells that appear to belong to Group IV-VII have dendritic architectures similar to those described by Thanos et al. but with smaller dendritic areas. The growth of the somata and dendritic fields of ganglion cells is shown as a function of time in figure 5.9. It is possible that the dendritic expansion of the cells of Group I-III, which have smaller dimensions, is complete by E14, while the expansion of those belonging to Group IV-VII cell types with more extensive fields, continues after E14. Unfortunately, it proved very difficult to apply the whole-cell patch-clamp technique to the retinae of older embryos, because removing small areas of the fibre layer at these later times was difficult and left debris that prevented the formation of a gigaseal between the patch pipette tip and the ganglion.
cell body membrane. The displaced ganglion cell type (Group VIII described by Thanos et al.) was not dye-filled in the studies described here, because the cell bodies of these neurons lie in the INL and were not accessible to patch-electrodes.

Chick Group I-III cells are similar in morphology to the β-ganglion cells of mammals and in the studies described have been shown to be dye-coupled to both other ganglion and amacrine cells. However, in the developing ferret retina and in the adult cat β-ganglion cells are not dye-coupled (Penn et al., 1994; Boycott and Wässle, 1974). Similarly, I have shown that during development what appear to be Group V (α-like) and Group VII (γ-like) chick ganglion cells are coupled to both other ganglion and amacrine cells. While it is hard to be certain of the subtype of the ganglion cells to which the injected cell is coupled, because they were usually too weakly stained to identify them unequivocally, differences in size and shape of the somata of the coupled cells suggest that coupling is both, homologous and heterologous. Heterologous coupling was not seen by Penn et al. (1994) nor by Vaney et al. (1991). Thus the patterns of coupling seen during development of the chick retina may either be transitional toward, or representative of, the adult pattern in chicken which has unfortunately yet to be determined. That the coupling patterns seen are an artefact seems unlikely, since coupling was abolished by application of the gap junction blocker octanol.

5.4.3 Modulation of retinal gap junctions in development

The spontaneous Ca\(^{2+}\)-transients seen in the chick retina are modulated by dopamine (Catsicas et al., 1998). Low doses of dopamine (100 nM) increase the frequency of the transients while high doses (50-100 μM) decrease them. The dopamine uptake blocker 3a-bis-(4-fluorophenyl)-methoxytropane has a similar action increasing transiently the frequency at early times and decreasing it with longer application times. Since there is an extensive
network of gap junctions between retinal ganglion cells and amacrine cells at the times when these Ca$^{2+}$-transients occur and because their frequency is modulated by dopamine, which is a well known gap junctional modulator in the adult retina, I examined the effects of dopamine on the dye-coupling of ganglion cells at E11.

As shown in figure 5.13 dopamine had no significant effect on the dye-coupling of ganglion cells. This suggests that the effect of dopamine on the frequency of spontaneous Ca$^{2+}$-transients (Catsicas et al., 1998) must be due to modulation of gap junctions other than those shown here or else results from dopamine’s actions on neuronal excitability. Dopamine receptor activation can both reduce K$^+$-permeability and voltage-dependent Ca$^{2+}$-currents (Yan et al., 1997; vanGoor et al., 1998; Wilke et al., 1998).

5.5 Further experiments

It would be interesting to determine the pattern of ganglion cell dye-coupling at later times in development and in the adult chick retina. This might be possible using the mixture of fluorescein dextran and Neurobiotin employed here and sharp electrodes to show whether or not the pattern of coupling reported here are transitional towards that seen in the adult. The importance of gap junctional communication for the formation of the appropriate synaptic contacts could be investigated using antisense technology to determine whether the reduction seen in gap junctional communication at the beginning of synaptogenesis is required for the proper wiring of the retina.
Conclusions

I have investigated the presence of amino acid transmitter systems and gap junctions as a means of communication between developing neurons in the embryonic chick retina. The amino acids glutamate, aspartate and glycine, unlike ACh and other transmitters, are important metabolites that are present in the cell from earliest times. The presence of these amino acids early on raises the possibility that they are important before the machinery for their release becomes localised at synapses.

The HPLC experiments reported here show that the Ca\textsuperscript{2+}-dependent component of depolarisation-evoked transmitter release is greater when synapses are formed than at early times. However, the amount of glutamate, aspartate and glycine present in the extracellular medium under control conditions was higher at early times in development than at the onset of synaptogenesis (except for GABA, see below). The presence of these amino acids in the extracellular space at such early times highlights the possibility that they have important developmental roles prior to synapse formation.

GABA, which like glycine depolarises neurons early in development due to the high [Cl\textsuperscript{-}], (Owens et al., 1996), was released only in very small amounts early in retinogenesis. Since GAD is thought to be absent at early times, the GABA present in cells at these times may be synthesised from putrescine. GABA release has been reported from isolated growth cones in rat brain preparations (Taylor and Gordon Weeks, 1989). Since very little GABA is released during early development and GABA receptors are present on ganglion cells and other retinal neurons it is possible that they are activated by another ligand such as glycine or taurine.

The high levels of glycine in the extracellular space of the retina at early times may thus play roles similar to that of GABA which in the cortex has
been shown to depolarise ventricular zone cells and may cause them to leave the cell cycle through a depolarisation-evoked increase in $[\text{Ca}^{2+}]_i$ (LoTurco et al., 1995). However, if this is so it is curious that imaging experiments have failed to detect such changes in $[\text{Ca}^{2+}]_i$ in response to application of glycine between E4 and E13.

When the results of the HPLC analysis of amino acid release are compared with the developmental profile of the appearance of amino acid transmitter receptors, it at first seems surprising that the extracellular concentrations of glutamate, aspartate and glycine are high at early times, while at the same time the number of ganglion cells expressing the appropriate receptors is low and that while receptors appear early, the release of GABA could not be detected until later. However, it is likely that while GC's do not express receptors for glutamate or glycine at early times, other cells, perhaps within the ventricular zone, do possess these receptors.

While it is known that ventricular zone cells are extensively dye-coupled during early development of the CNS (Peinado et al., 1993), the youngest ganglion cells that were filled with dye at E5 showed hardly any coupling to other cells. The sites of potential coupling between ganglion and other cells at early times were dendro-somatic, dendro-axonic and dendro-dendritic. At later times, the contact points that may represent the location of gap junctions between the coupled cells were exclusively dendro-dendritic. It is likely that new-born ganglion cells loose any gap junctional partners they may have had in the ventricular zone and find new ones once they reach the GCL. The formation of gap junctions by ganglion cells seems to coincide with the elaboration of dendrites, since it is not until the beginning of process outgrowth that dye-coupling becomes extensive. Dye-coupling is greatest at times prior to synapse formation and subsequently decreases. This result is similar to that obtained by Kandler and Katz (1998a) in the cortex. It is unknown whether or not gap junctional connections play any part in specifying future synaptic connections between neurons.
In summary, it appears that both amino acid transmitters and gap junctions are employed extensively as a means of intercellular communication during development of the chick retina. The pattern of appearance of transmitter receptors, transmitter release and gap junctions is complex. It is likely that both chemical and gap junctional communication play changing roles during development even for individual cell types.
References


